

Potentiation of acid-sensing ion channels by sulfhydryl compounds

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Submitted 1 December 2006; accepted in final form 15 February 2007

Cho J-H, Askwith CC. Potentiation of acid-sensing ion channels by sulfhydryl compounds. *Am J Physiol Cell Physiol* 292: C2161–C2174, 2007. First published March 28, 2007; doi:10.1152/ajpcell.00598.2006.—The acid-sensing ion channels (ASICs) are voltage-independent ion channels activated by acidic extracellular pH. ASICs play a role in sensory transduction, behavior, and acidotoxic neuronal death, which occurs during stroke and ischemia. During these conditions, the extracellular concentration of sulfhydryl reducing agents increases. We used perforated patch-clamp technique to analyze the impact of sulfhydryls on H⁺-gated currents from Chinese hamster ovary (CHO) cells expressing human ASIC1a (hASIC1a). We found that hASIC1a currents activated by pH 6.5 were increased almost twofold by the sulfhydryl-containing reducing agents dithiothreitol (DTT) and glutathione. DTT shifted the pH-dose response of hASIC1a toward a more neutral pH (pH_{0.5} from 6.54 to 6.69) and slowed channel desensitization. The effect of reducing agents on native mouse hippocampal neurons and transfected mouse ASIC1a was similar. We found that the effect of DTT on hASIC1a was mimicked by the metal chelator TPEN, and mutant hASIC1a channels with reduced TPEN potentiation showed reduced DTT potentiation. Furthermore, the addition of DTT in the presence of TPEN did not result in further increases in current amplitude. These results suggest that the effect of DTT on hASIC1a is due to relief of tonic inhibition by transition metal ions. We found that all ASICs examined remained potentiated following the removal of DTT. This effect was reversed by the oxidizing agent DTNB in hASIC1a, supporting the hypothesis that DTT also impacts ASICs via a redox-sensitive site. Thus sulfhydryl compounds potentiate H⁺-gated currents via two mechanisms, metal chelation and redox modulation of target amino acids.

glutathione; DTT; redox; zinc

ACID-SENSING ION CHANNELS (ASICs) are members of the degenerin (DEG)/epithelial Na channel (ENaC) ion channel family. These voltage-independent ion channels are activated by extracellular acidosis and are expressed in neurons throughout the central and peripheral nervous system (46, 47). There are four ASIC genes that encode at least six ASIC subunits (ASIC1a, -1b, -2a, -2b, -3, and -4). ASIC subunits share common structural features with all DEG/ENaC ion channels including an intracellular NH₂ and COOH terminus, two transmembrane domains, and a large cysteine-rich extracellular region (27). Individual subunits associate to form homomultimeric or heteromultimeric channels with different characteristics (7, 10, 23). ASICs are cation permeable and modulate action potential generation in neurons upon extracellular acidification (34, 45). In the peripheral nervous system, disruption of individual ASIC genes affects multiple aspects of sensory transduction including mechanotransduction and nociception (13, 26, 31, 35, 36). In the central nervous system, disruption of ASIC1 impacts fear-related behaviors as well as learning and memory (50–52).

ASICs contribute to neuronal death following stroke and ischemia (55). During these conditions, the extracellular environment in affected tissue becomes acidic (37). This increase in proton concentration is thought to activate ASICs. In the brain, many neurons express both ASIC1a homomultimeric and ASIC1a/2a heteromultimeric channels (7, 17). Homomultimeric ASIC1a channels are calcium permeable and require less acidic pH to be activated than ASIC1a/2a channels (7, 9, 16, 56). Activation of ASIC1a channels causes an acute increase in intracellular calcium. Excess activation of ASIC1a channels during stroke and ischemia causes neuronal death (55, 56). Injection of venom containing PcTX1, a peptide known to prevent ASIC1a activation, reduces neuronal damage in mouse models of ischemia (55). Additionally, mice with a genetic disruption of the ASIC1 gene show less damage following ischemia (55). These results indicate that preventing ASIC activation limits damage, and, therefore, agents that alter ASIC activity may impact acidosis-induced neuronal death following stroke. ASICs also impact migration of malignant glioma cells, suggesting that ASIC activity may also play a role in the pathogenesis of brain tumors (11, 44).

ASICs are modulated by several compounds in a subunit-specific manner. For example, RFamide-related peptides slow or prevent desensitization of ASIC1- or ASIC3-containing channels (6, 12, 14, 19, 32, 54). Low concentrations of zinc limit ASIC1a and ASIC1a/2a channel activation, while high concentrations potentiate ASIC2a homomultimeric channels (8, 17, 21). Recent work indicates that rodent ASIC1a activity is also affected by redox reagents (5, 15). These studies reported different effects of the redox reagents and suggested distinct mechanisms of sulfhydryl-induced modulation. To gain insight into the mechanism of modulation of human and mouse ASICs (hASICs and mASICs, respectively), we undertook a study of glutathione and dithiothreitol (DTT), an exogenous sulfhydryl reagent that mimics the effects of endogenous sulfhydryls. Our results suggest that sulfhydryls potentiate ASIC1a through two mechanisms: reducing amino acid residues within the ASIC protein and chelating transition metals that tonically inhibit ASIC activity. Furthermore, we found that sulfhydryl compounds induce potentiation of most ASICs. These results lend significant insight into sulfhydryl modulation of ASICs and suggest that sulfhydryl-induced potentiation may impact neuronal activities linked to ASIC function.

MATERIALS AND METHODS

DNA constructs and Chinese hamster ovary cell transfection. Human and mouse ASIC cDNA constructs were cloned in pMT3 expression vector as described (7). The K133R mutant of hASIC1a was made using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Chinese hamster ovary (CHO) cells were

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transiently transfected with ASIC constructs using electroporation. Briefly, trypsinized CHO cells ($\sim 10^7$ cells) were suspended in 0.4 ml of electroporation solution (120 mM KCl, 25 mM HEPES, 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 2 mM $MgCl_2$, 0.15 mM $CaCl_2$, 5 mM EGTA, and 2 mM MgATP, pH 7.6) and mixed with 2–4 μ g of pEGFP-C1 (Clontech, Mountain View, CA) as well as 10–20 μ g of ASIC constructs. Cells were electroporated with the Gene Pulser Xcell system (Bio-Rad Laboratories, Hercules, CA) and plated at a density of 35 cells/mm² onto 10-mm coverslips in a 35-mm culture dish. Cells were used for patch-clamping 2–3 days after transfection. Transfected cells were identified by green fluorescent protein (GFP) fluorescence.

Primary neuronal culture. Primary hippocampal neuron cultures were prepared using previously published methods (7, 51). Briefly, hippocampi were dissected from postnatal day 0–2 pups, freed from extraneous tissue, and cut into approximately eight pieces. The hippocampal tissue was transferred into Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) containing 0.25 mg/ml bovine serum albumin and 0.375 mg/ml papain and incubated for 15 min at 37°C with 95% O₂-5% CO₂ gently blown over the surface of the medium. After incubation, the hippocampal tissue was washed three times with mouse M5-5 medium (Earle's minimal essential medium with 5% fetal bovine serum, 5% horse serum, 0.4 mM L-glutamine, 22 mM glucose, penicillin-streptomycin, and insulin-selenite-transferrin) and triturated. Hippocampal cells (5×10^5 cells/well) were plated onto collagen-coated 10-mm coverslips in 24-well culture dishes. After 72 h, cytosine β -D-arabinofuranoside was added to inhibit glial proliferation. After 10 days in vitro, one-half of the culture medium was replaced with fresh M5-5 medium every 3 days. Neurons were used from 12 to 20 days in culture.

Electrophysiology. We used the nystatin-based perforated patch-clamp technique to record H⁺-gated currents. This method proved very stable, and ASIC current rundown was significantly smaller in perforated patch recording compared with conventional whole cell patch-clamping. The extracellular solution contained 140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 10 mM MES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.55 mM glucose. Tetramethylammonium hydroxide was used to adjust the pH of the extracellular solution to pH 4.0–7.4. The intracellular pipette solution contained 130 mM K-gluconate, 20 mM KCl, 10 mM HEPES, and 0.1 mM EGTA (pH 7.3). The pipette tip was filled with the intracellular solution and then back-filled with the solution containing 150 μ g/ml nystatin. Nystatin stock solution (30 mg/ml) was made fresh in DMSO before patch-clamping. Patch electrodes were pulled with a P-97 micropipette puller (Sutter Instrument, Novato, CA) and fire-polished with a microforge (Narishige, East Meadow, NY). Micropipettes with 3–7 M Ω were used for experiments. Large pyramidal neurons were chosen for patch-clamp analysis. After attaining a giga-ohm seal, we monitored whole cell membrane capacitance and series resistance until the patch was fully perforated by nystatin. This usually occurred within 5 min. Cells were continuously superfused with the extracellular solution from gravity-fed perfusion pipes at a flow rate of ~ 1 ml/min. Perfusion pipes were placed 250–300 μ m away from cells, and flow was directed toward the recorded cells to ensure fast solution exchange. For hippocampal neurons, we added ion channel inhibitors to the extracellular solution to inhibit synaptic currents (10 μ M cyano-7-nitroquinoxaline-2,3-dione, 50 μ M D-2-amino-5-phosphonovaleic acid, 30 μ M bicuculline, and 500 nM tetrodotoxin). The addition of DTT, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), or *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) did not alter the pH of the extracellular solution. Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA).

The membrane potential was held constant at -70 mV. Data were collected at 5 kHz using an Axopatch 200B amplifier, Digidata 1322A, and Clampex 9 (Molecular Devices, Sunnyvale, CA). In most experiments, H⁺-gated currents were evoked by the exogenous ap-

plication of pH 6.5 or 6.0 extracellular solutions at 2–2.5-min intervals. Because of current rundown, we evoked four to five H⁺-gated currents to ensure a stable pretreatment control value before the incubation of reagents. Data were analyzed using Clampfit 9 software (Molecular Devices). Maximal current amplitudes were normalized to the pretreatment control values and are expressed as means \pm SE. For determining the pH dose-response curves, peak current amplitudes at different test pHs were normalized to the average amplitude of pH 5.0 currents evoked just before and after the test pH application. The average of normalized currents at different pHs was fitted to the equation

$$I/I_{pH5.0} = 1/\{1 + (EC_{50}/[H^+])^n\} = 1/\{1 + 10^{n(pH-pH_{0.5})}\}$$

where n is Hill coefficient, and EC_{50} and $pH_{0.5}$ are the proton concentration and pH yielding one-half of the pH 5.0 currents ($I_{pH5.0}$), respectively. To ensure that ASIC1a/2a heteromultimers were generated by expression of both ASIC1a and ASIC2a in CHO cells, we measured the rate of recovery from desensitization. Briefly, pH 6.0 was applied for 3–5 s, the pH was returned to 7.4 for ~ 2.5 s, and then a second pH 6.0 application was made. We calculated the percent recovery by comparing the peak amplitude of the first pH 6.0 application to that of the second pH 6.0 application. Consistent with previous reports (7, 10), the recovery rate was faster in cells expressing both mASIC1a and mASIC2a ($74 \pm 4\%$, $n = 13$) than in cells expressing mASIC1a alone ($41 \pm 3\%$, $n = 13$, $P < 0.0001$, unpaired *t*-test). We used unpaired Student's *t*-test for the comparison of H⁺-gated currents from different groups of cells and the paired Student's *t*-test when comparing H⁺-gated currents with the pretreatment control in the same cell. We used one-way ANOVA to compare currents among more than two different groups. Statistical analyses were performed with Minitab14 software (Minitab, State College, PA).

RESULTS

DTT potentiates homomeric hASIC1a currents in CHO cells.

To better understand the mechanism of sulfhydryl modulation of ASIC currents, we expressed hASIC1a in CHO cells and used perforated patch-clamp to analyze whole cell currents. We evoked H⁺-gated currents by changing the extracellular pH from 7.4 to 6.5 for 5 s. Once stable pH 6.5-evoked currents were established, we exposed cells to 1 mM DTT at pH 7.4. One minute after the addition of DTT, we activated hASIC1a by changing the pH from 7.4 to 6.5 in the presence of DTT (Fig. 1A). We found that the peak amplitude of H⁺-gated currents was increased almost twofold in the presence of 1 mM DTT compared with currents recorded before DTT addition ($182 \pm 17\%$ of pre-DTT control, $n = 18$, $P = 0.0009$; Fig. 1, B–C). ASIC desensitization was also dramatically slowed in the presence of DTT (Fig. 1A). Similar results were obtained with 10 μ M DTT (peak amplitude $180 \pm 20\%$ of pre-DTT control, $n = 10$, $P = 0.003$; Fig. 1C), indicating that lower concentrations of DTT have similar effects. Both pH 6.5 and DTT had no effect on CHO cells expressing only GFP (Fig. 1D), indicating that the potentiation observed was dependent on hASIC1a expression. Thus DTT potentiates hASIC1a currents.

The potentiation by DTT could be due to two mechanisms, chelation of transition metals and/or reduction of amino acid residues (33, 42). The first effect would be transient and persist only as long as DTT is present. The latter effect could endure after the removal of DTT because of the stable nature of covalent modification. To investigate the involvement of these two mechanisms in sulfhydryl modulation of hASIC1a, we

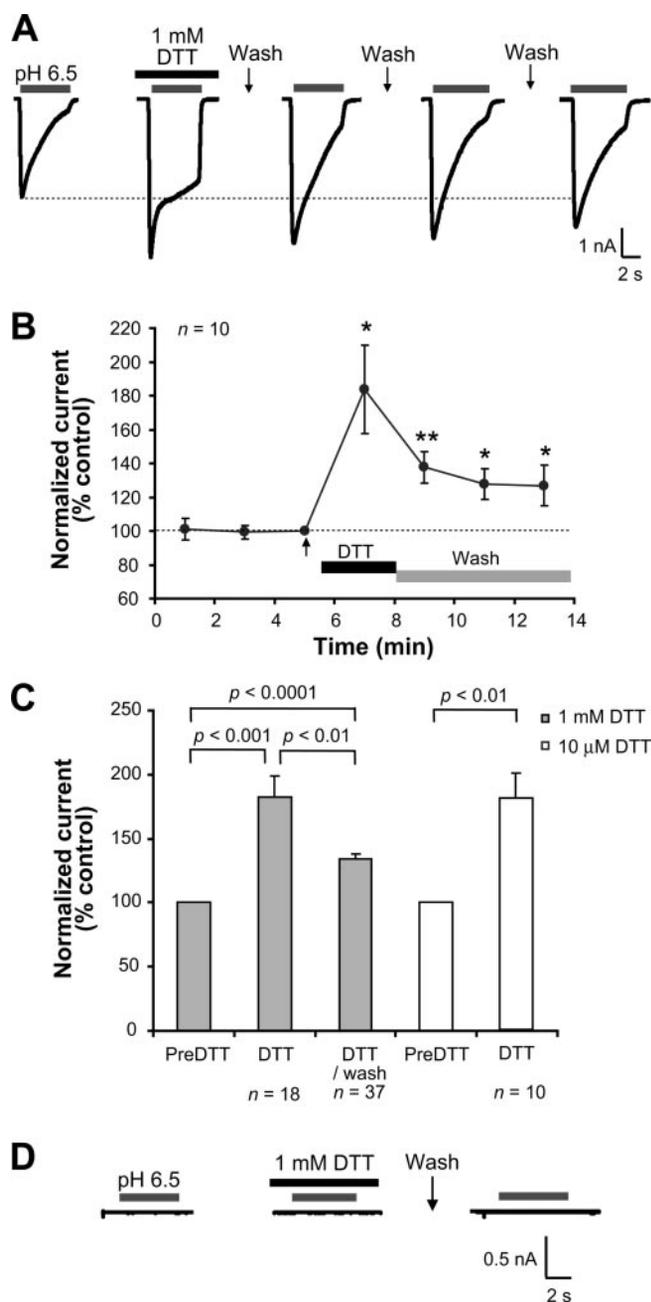


Fig. 1. DTT potentiates H^+ -gated currents in Chinese hamster ovary (CHO) cells expressing human acid-sensing ion channel 1a (hASIC1a). **A**: representative trace of H^+ -gated currents before and after DTT incubation. CHO cells expressing hASIC1a were exposed to extracellular acid (pH 6.5, gray bars) and 1 mM DTT for 3 min (black bar). DTT-free solution exchange for 1–5 min is indicated by “wash” arrows. Dotted line represents the peak current amplitude before DTT addition. **B**: time course of DTT-induced potentiation of hASIC1a H^+ -gated current. Extracellular pH 6.5 solutions were added at 2-min intervals. Peak current amplitude was normalized to that of the pre-DTT control (100%, indicated by arrow) evoked just before DTT incubation ($n = 10$). * $P < 0.05$ and ** $P < 0.01$ compared with control (paired t -test). **C**: quantification of the transient (DTT) and long-lasting effects (DTT/wash) of 1 mM and 10 μ M DTT on pH 6.5-activated currents. Data are expressed as the mean of normalized peak current amplitude before addition of DTT, in the presence of DTT, and following wash with DTT-free solution (1 min). P values were determined using a paired t -test. **D**: representative trace of the effect of DTT and pH 6.5 in CHO cells transfected with pEGFP alone. Note that H^+ -gated current was not evoked by acid (pH 6.5), and DTT incubation had no effect. In all experiments, error bars represent SE.

assessed whether currents remained potentiated after the removal of DTT. Cells were incubated with DTT solution at pH 7.4 for 3 min and then superfused with DTT-free pH 7.4 solution for 1 min. Following this wash step, the pH was changed to pH 6.5 in the absence of DTT to activate ASIC1a currents (Fig. 1A). The amplitude of pH 6.5 currents after DTT incubation and removal was $134 \pm 4\%$ of the pre-DTT control ($n = 37$, $P < 0.0001$; Fig. 1, A–C). This potentiation was significantly smaller than the potentiation observed in the presence of DTT ($n = 13$, $P = 0.005$). We continued to superfuse the cells with pH 7.4 solution and activate ASIC1a currents with pH 6.5 every 2 min. We found that the increase in peak current amplitude persisted for at least 5 min following DTT removal (Fig. 1, A–B). In cells that afforded analysis at later times after DTT incubation, we found that the effect was maintained for as long as 15 min ($n = 2$, data not shown). Together, these results indicate that DTT has both transient (the increase in current in the presence of DTT) and long-lasting (the potentiation after DTT removal) effects on hASIC1a.

We hypothesized that the long-lasting potentiation of hASIC1a currents after DTT removal may be due to reduction of specific amino acids by DTT. To determine whether oxidation of those residues could reverse the effect of DTT, we incubated the cells with DTNB, a compound that covalently modifies proteins and forms mixed disulfides with free sulfhydryl groups (Fig. 2). Following DTT incubation and removal, hASIC1a currents were activated by pH 6.5 solution. Then, DTNB was applied for 3 min and washed away for an additional 3 min (Fig. 2A). After DTNB incubation and removal, hASIC1a currents were again activated by changing the pH from 7.4 to 6.5. We found that DTNB exposure reversed the long-lasting potentiation of hASIC1a currents following DTT incubation and washout ($130 \pm 5\%$ after DTT/wash and $108 \pm 5\%$ after DTNB/wash, $n = 12$; Fig. 2B). Without previous DTT exposure, the application and removal of DTNB did not alter the amplitude of H^+ -gated currents ($107 \pm 5\%$ of previous pre-DTNB control, $n = 9$, $P = 0.24$; Fig. 2, C–D). Therefore, the effect of DTNB was dependent on previous exposure to DTT. These results indicate that DTT-induced reduction of residues within hASIC1a potentiates H^+ -gated currents, and oxidation of these reduced residues by DTNB reverses this potentiation.

Together, our results indicate that DTT has two effects on hASIC1a. First, the presence of DTT during ASIC activation induces large potentiation of pH 6.5-activated hASIC1a currents. Once DTT is removed, potentiation is reduced, but current amplitude remains increased compared with pre-DTT controls. This effect is reversed by DTNB, indicating that reduction of amino acid residues by DTT is required for this long-lasting effect.

Glutathione potentiates hASIC1a. DTT is an exogenous sulfhydryl compound. To test whether endogenous sulfhydryl compounds could potentiate hASIC1a, we treated transfected CHO cells with reduced glutathione. In the presence of 100 μ M glutathione, the amplitude of H^+ -gated currents increased ($165 \pm 16\%$, $n = 9$, $P = 0.003$; Fig. 3, A–B). Similar to DTT treatment, the amplitude of H^+ -gated currents decreased partly after a 5-min washout but still remained elevated compared with preglutathione control ($129 \pm 9\%$, $n = 7$, $P = 0.02$; Fig. 3, A–B). We also tested the effects of glutathione at lower

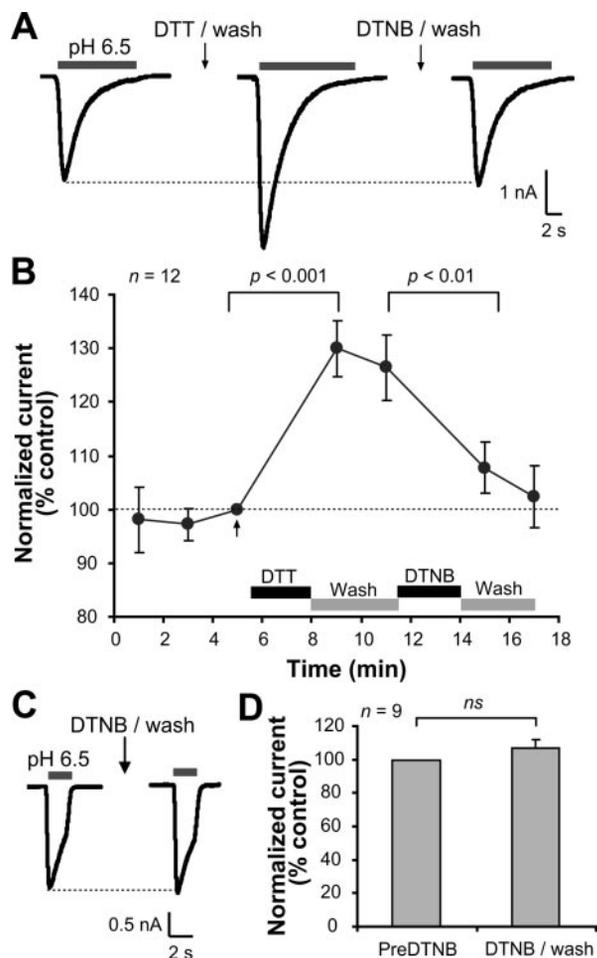


Fig. 2. The long-lasting effect of DTT on hASIC1a is reversed by DTNB. **A:** representative trace of the effect of DTT and DTNB on hASIC1a expressed in CHO cells. H^+ -gated currents were activated by pH 6.5 solution before DTT addition, after DTT addition (1 mM DTT for 3 min and 3-min wash), and after DTNB addition (1 mM DTNB for 3 min and 3-min wash). Note that all pH 6.5 applications were performed in the absence of DTT or DTNB. **B:** time course of the long-lasting effects of DTT and DTNB. Peak current amplitude was normalized to that of the pre-DTT control (arrow). DTT and DTNB additions are indicated by black bars. Washes with extracellular solution free of reagents are indicated by gray bars ($n = 12$, paired t -test). **C:** representative trace of the effect of DTNB (1 mM) on hASIC1a currents without previous DTT incubation. DTNB was added for 3 min and washed out for 3 min. **D:** quantification of the effect of DTNB alone on hASIC1a. pH 6.5-activated current amplitude from hASIC1a was not significantly affected (ns) by DTNB when cells were not previously treated with DTT ($n = 9$, $P = 0.24$, paired t -test). Error bars represent SE.

concentrations (1 μ M and 10 nM; Fig. 3B) and observed that potentiation in the presence of glutathione was concentration dependent (Fig. 3C). Persistent potentiation that remained after the removal of glutathione was only observed with 100 μ M and 1 μ M glutathione, suggesting that the long-lasting potentiation requires higher concentrations of glutathione (micromolar range), whereas transient potentiation in the presence of glutathione requires much lower concentrations of glutathione (nanomolar range).

DTT affects hASIC1a pH dose-response and channel desensitization. Our results indicate that DTT impacts the peak current amplitude of pH 6.5-activated hASIC1a currents and channel desensitization (Fig. 1A). hASIC1a channels desensi-

tize in the presence of acidic solution, and desensitization controls the duration of ASIC currents. The presence of DTT caused an obvious slowing of channel desensitization (Figs. 1A and 4A). Desensitization is usually quantified by fitting the desensitization phase of the ASIC current to a single exponential equation and calculating the tau of desensitization (τ_d).

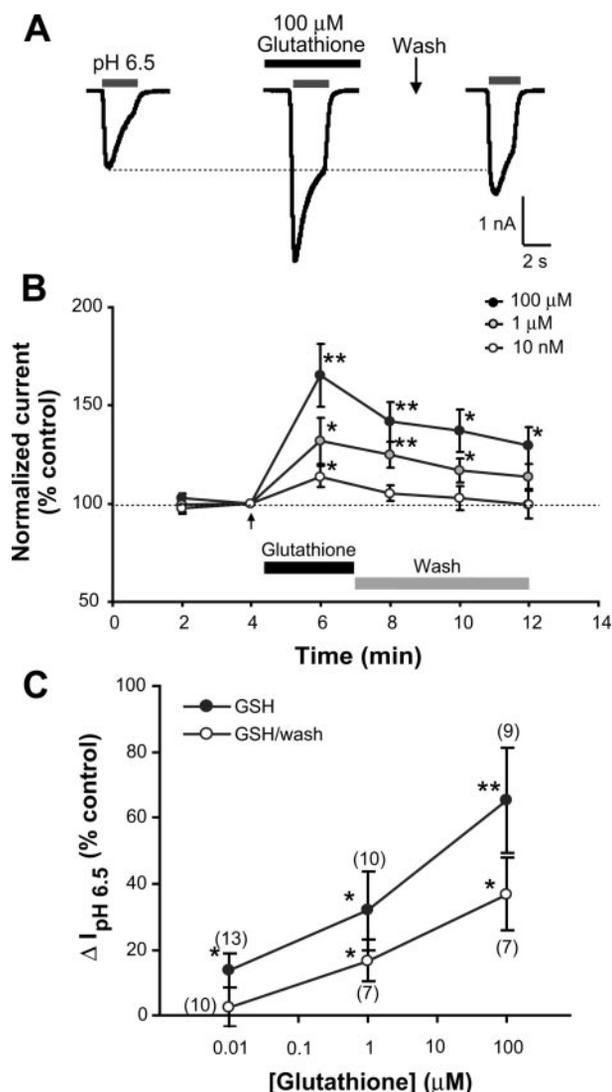


Fig. 3. Glutathione potentiates hASIC1a currents in a concentration-dependent manner. **A:** a representative trace shows the potentiation of hASIC1a current by reduced glutathione. H^+ -gated currents were activated by pH 6.5 solutions (gray bar) during glutathione incubation (100 μ M for 3 min as indicated by black bar) and following removal of glutathione (1-min wash). Washout is indicated by arrow. **B:** time course of the potentiation by 100 μ M, 1 μ M, and 10 nM glutathione. Peak amplitudes were normalized to preglutathione control (arrow). Glutathione incubation is indicated by black bar. Solution exchange (wash) is indicated by gray bar ($n = 7$ –13 for each group). $*P < 0.05$ and $**P < 0.01$ compared with control (paired t -test). **C:** concentration-response relationship of glutathione. The changes of current amplitudes ($\Delta I_{pH 6.5}$) during glutathione incubation (GSH) and after 3-min washout (GSH/wash) are expressed as the percentage of preglutathione control. $*P < 0.05$ and $**P < 0.01$ compared with control (paired t -test). A 1-way ANOVA revealed a significant difference between $\Delta I_{pH 6.5}$ during glutathione incubation and $\Delta I_{pH 6.5}$ after washout of glutathione [$F_{(1, 54)} = 4.49$, $P = 0.04$]. Both transient and long-lasting effects of glutathione on $\Delta I_{pH 6.5}$ were significantly different depending on the concentration [1-way ANOVA, $F_{(2, 29)} = 5.83$, $P = 0.007$ for the transient effect; $F_{(2, 21)} = 3.61$, $P = 0.04$ for the long-lasting effect]. Nos. in parentheses represent nos. of cells examined.

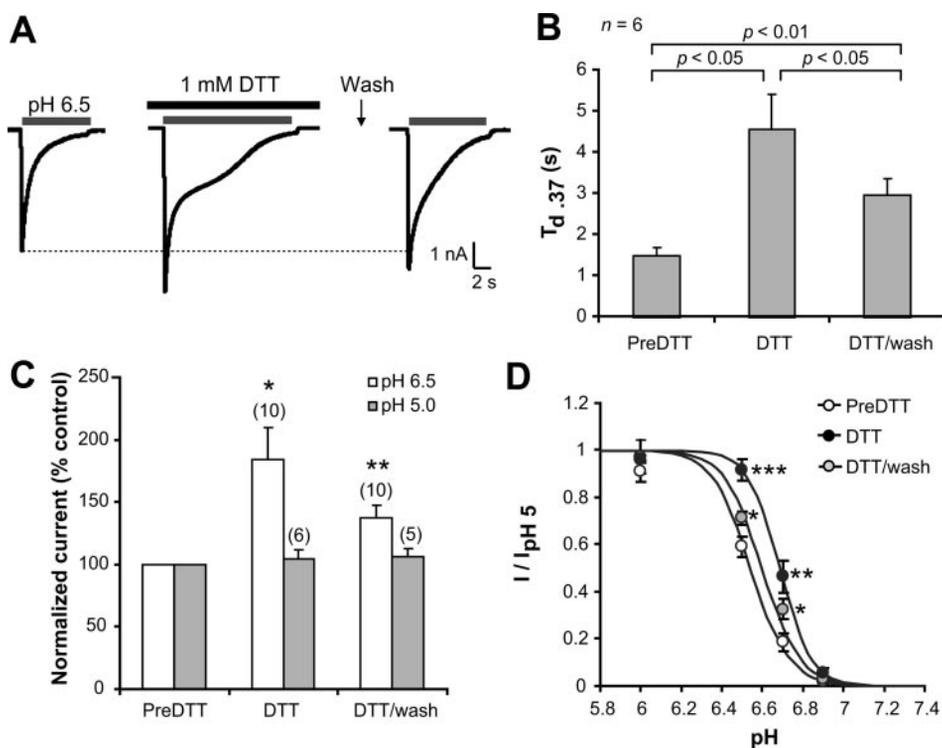


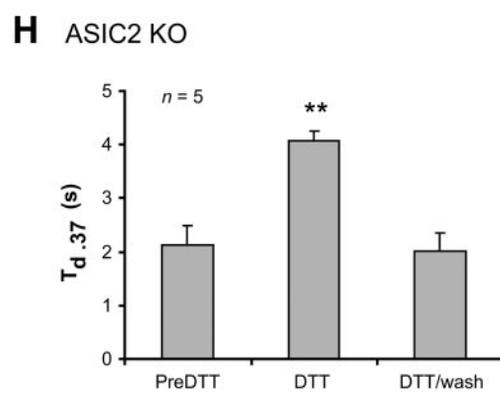
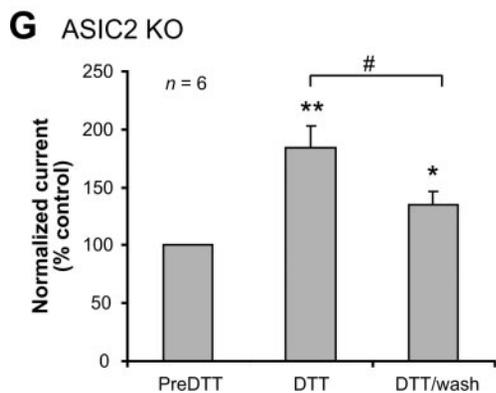
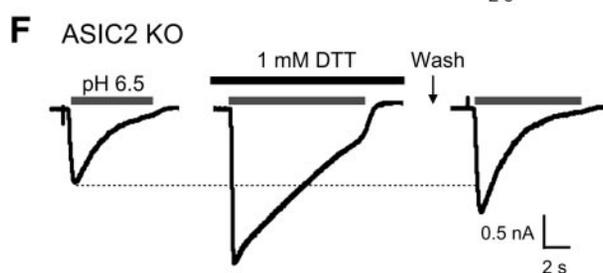
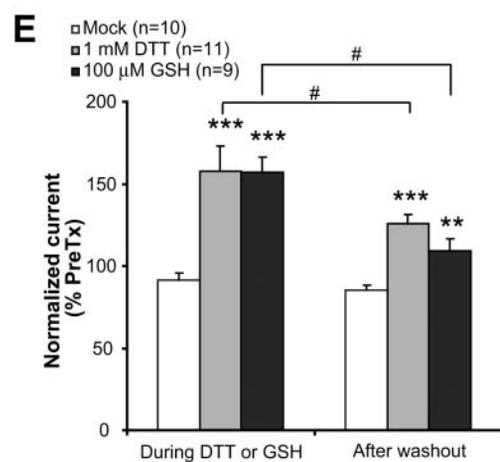
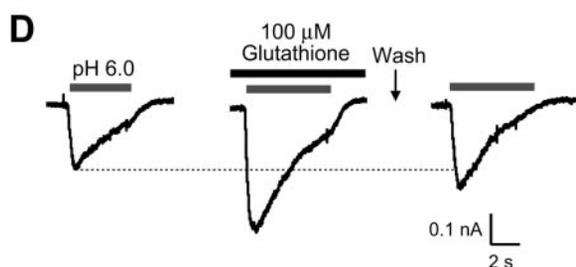
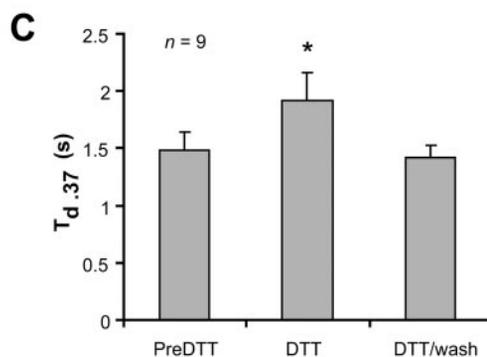
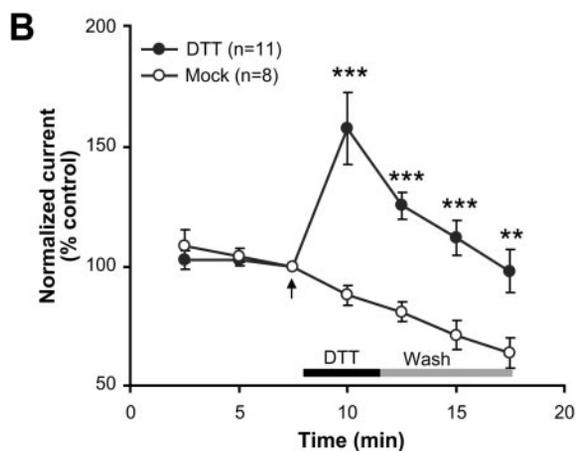
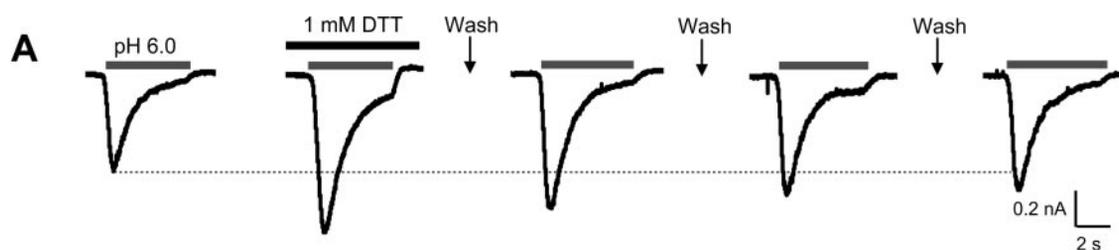
Fig. 4. DTT slows desensitization and shifts the pH dose response of hASIC1a. *A*: representative recording showing desensitization kinetics before, during, and after DTT incubation. pH 6.5 addition is indicated by gray bars, and DTT incubation (1 mM) is indicated by black bars. Removal of DTT-containing solution is indicated by wash arrow. Dotted line represents the peak current amplitude before DTT application. *B*: quantification of the DTT-induced change in desensitization. Desensitization was quantified as the time for hASIC1a currents to decrease to 37% of the peak amplitude ($T_{d,37}$). Statistical significance was determined using a paired *t*-test. Error bars represent SE. *C*: quantification of the peak current amplitude of pH 6.5- or pH 5.0-activated currents before, during, and after DTT addition. Peak current amplitudes were normalized to the pre-DTT control values. Nos. in parenthesis represent nos. of cells examined. * $P < 0.05$ and ** $P < 0.01$ compared with pre-DTT control (paired *t*-test). *D*: change in pH dose-response relationship of hASIC1a by DTT. H^+ -gated currents were evoked by pH 6.0, 6.5, 6.7, and 6.9 solutions without DTT incubation (pre-DTT), during DTT incubation (DTT), or after the washout of DTT (DTT/wash). Peak current amplitudes were normalized to currents evoked by pH 5.0 at the indicated condition ($n = 4-8$ for each data point, presented as mean \pm SE). Normalized currents at pH 6.5 and 6.7 were significantly larger in DTT-treated cells compared with cells without DTT incubation. I , peak current amplitude at given pH values; $I_{pH 5.0}$, peak current amplitude at pH 5.0. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (unpaired *t*-test).

This point represents the time required for the current to reduce to 37% of its maximal value ($1/e$). Since hASIC1a desensitization in the presence of DTT did not fit to an exponential equation, we quantified desensitization by measuring the time required for H^+ -gated currents to decrease to 37% of the peak amplitude ($T_{d,37}$). Using this method to quantify desensitization, we found that the $T_{d,37}$ of hASIC1a currents at pH 6.5 was larger in the presence of DTT ($T_{d,37} = 1.49 \pm 0.21$ s for the pre-DTT control, 4.56 ± 0.86 s during DTT incubation, $n = 6$, $P = 0.011$; Fig. 4*B*). After the removal of DTT, desensitization of H^+ -gated currents was still twofold larger than pre-DTT control values ($T_{d,37} = 2.97 \pm 0.40$ s after DTT washout, $n = 6$, $P = 0.007$; Fig. 4*B*). These results indicate that desensitization of hASIC1a was slowed in the presence of DTT and remained affected after washout of DTT.

Our data show that DTT potentiates hASIC1a currents activated by pH 6.5 solutions (Fig. 1). To determine whether DTT potentiation was dependent on the pH of the activating solutions, we analyzed DTT effects using different activating pHs. We found that currents activated by pH 5.0 did not change significantly during or after DTT incubation ($105 \pm 7\%$, $n = 6$, $P = 0.41$ during DTT incubation; $106 \pm 6\%$, $n = 5$, $P = 0.91$ after washout of DTT; Fig. 4*C*). These results suggested that DTT-induced potentiation was pH dependent. We performed a detailed pH dose-response analysis of hASIC1a

during DTT incubation and after DTT removal. The pH that induced one-half maximal peak current amplitude ($pH_{0.5}$) of hASIC1a before DTT incubation was 6.54. During DTT incubation, the pH dose-response curve shifted toward neutral physiological pH with $pH_{0.5} = 6.69$ (Fig. 4*D*). Following DTT incubation and washout, H^+ -gated currents at pH 6.5 and 6.7 were still significantly elevated, and the pH dose-response curve remained shifted toward neutral pH with $pH_{0.5} = 6.60$ (Fig. 4*D*). These results indicate that DTT increases the apparent affinity of hASIC1a to protons, and that the effect of DTT is pH dependent.

Potentiation of ASIC currents by sulfhydryl compounds in hippocampal neurons. Our data indicate that sulfhydryls potentiate hASIC1a currents. To examine whether neuronal H^+ -gated currents are similarly affected by sulfhydryl compounds, we cultured hippocampal neurons from postnatal mice and tested the effects of DTT and reduced glutathione. Homomeric ASIC1a and heteromeric ASIC1a/2a channels contribute to H^+ -gated currents in central neurons (7). Because these two types of ASICs have different pH sensitivities, and the effect of DTT is pH dependent, we used pH 6.0 to activate H^+ -gated currents from neurons (Fig. 5*A*). Similar to our observations in CHO cells transfected with hASIC1a, the amplitude of pH 6.0-activated currents increased in the presence of 1 mM DTT ($158 \pm 15\%$, $n = 11$, $P = 0.004$; Fig. 5*A*). After removal of



DTT, currents remained elevated compared with pre-DTT control currents ($126 \pm 5\%$, $n = 11$, $P = 0.0007$; Fig. 5, A–B). The peak current amplitudes of H⁺-gated currents in hippocampal neurons consistently decrease with repeated applications of acidic solutions. Therefore, we compared DTT-treated neurons to mock-treated neurons and observed that DTT-treated neurons showed larger currents for at least 6 min, indicating that potentiation persisted even after the removal of DTT (Fig. 5B). H⁺-gated current desensitization was slowed in the presence of DTT ($T_{d.37} = 1.45 \pm 0.16$ s for pre-DTT control, 1.94 ± 0.30 s during DTT incubation, $n = 9$, $P = 0.033$; Fig. 5C). As opposed to our results in CHO cells, however, current desensitization after the removal of DTT was not significantly different from pre-DTT control values ($T_{d.37} = 1.42 \pm 0.11$ s, $n = 9$, $P = 0.37$; Fig. 5C). We also tested the effect of the endogenous sulfhydryl compound glutathione. Like DTT, glutathione affected H⁺-gated current amplitude (Fig. 5D). Quantification revealed that glutathione and DTT induced similar potentiation (Fig. 5E).

To determine whether the differences in DTT modulation between hASIC1a expressed in CHO cells and H⁺-gated currents in mouse neurons were due to the presence of ASIC2 subunits, we analyzed DTT effects in neurons from ASIC2 knockout mice. In hippocampal neurons, H⁺-gated currents are due to activation of both homomultimeric ASIC1a channels and heteromultimeric ASIC1a/2a channels. In the absence of the ASIC2, ASIC1a homomultimeric channels predominate (7). We found that H⁺-gated currents in ASIC2 knockout neurons were potentiated in the presence of DTT and remained potentiated following the removal of the sulfhydryl reagent ($184 \pm 19\%$, $P = 0.008$ during DTT incubation; $135 \pm 11\%$, $P = 0.023$ after DTT incubation and washout, $n = 6$; Fig. 5, F–G). The $T_{d.37}$ was also increased in the presence of DTT but returned to pre-DTT levels with the removal of DTT from solution (Fig. 5H). These results indicate that ASIC2 subunits are not responsible for the difference in DTT effects between CHO cells expressing hASIC1a and hippocampal neurons. This suggests that other factors such as species differences between mouse and human ASIC1a are responsible.

DTT potentiates mASICs in CHO cells. The presence of DTT affected H⁺-gated currents in mouse neurons differently from H⁺-gated currents in CHO cells expressing hASIC1a. To determine whether species differences between mouse and human ASIC1a impact DTT-induced potentiation, we analyzed the effects of DTT on mouse homomeric ASIC1a and heteromeric ASIC1a/2a expressed in CHO cells. Whereas DTT increased pH 6.5-activated hASIC1a currents twofold, mASIC1a pH 6.0-activated currents increased sevenfold in the presence of DTT ($716 \pm 137\%$ of pre-DTT control, $n = 9$, $P =$

0.002 ; Fig. 6A). After a 3-min DTT incubation and 1-min washout, DTT-induced effects decreased but currents remained potentiated compared with pre-DTT control ($199 \pm 33\%$ of pre-DTT control, $n = 9$, $P = 0.014$; Fig. 6A). The presence of DTT also slowed desensitization of mASIC1a ($T_{d.37} = 1.19 \pm 0.15$ s for pre-DTT control and 1.95 ± 0.35 s during DTT incubation, $n = 5$, $P = 0.021$; Fig. 6A). After removal of DTT, however, desensitization was not significantly different from pre-DTT control (1.36 ± 0.23 s, $n = 5$, $P = 0.39$). Therefore, species differences between mouse and human ASIC1a impact DTT-induced potentiation of ASIC1a currents.

We also analyzed the effects of DTT on mASIC1a/2a heteromultimeric channels (Fig. 6B). When DTT was applied to CHO cells expressing both mASIC1a and mASIC2a subunits, H⁺-gated currents increased twofold ($204 \pm 32\%$ of pre-DTT control, $n = 12$, $P = 0.0007$; Fig. 6B). Thus the effect of DTT on mASIC1a/2a pH 6.0-activated currents is substantially smaller than the effect of DTT on homomultimeric mASIC1a pH 6.0-activated currents ($P = 0.005$). After the removal of DTT, H⁺-gated currents remained increased, albeit to a much lesser extent ($128 \pm 9\%$ after the washout of DTT, $n = 12$, $P = 0.012$; Fig. 6B). Desensitization of mASIC1a/2a channels was also slowed during DTT incubation and after washout ($T_{d.37} = 1.20 \pm 0.08$ s for pre-DTT control, 1.88 ± 0.27 s during DTT incubation, $P = 0.027$, 1.51 ± 0.14 s after the washout, $P = 0.020$, $n = 7$; Fig. 6B).

DTT potentiation of H⁺-gated currents from other ASIC subunits was observed as well. We analyzed the effect of DTT on mASIC2a homomultimeric channels and found that DTT incubation caused an increase in mASIC2a current amplitude that persisted after the removal of DTT ($125 \pm 10\%$ of pre-DTT control, $n = 8$, $P = 0.038$; Fig. 7A). We also analyzed the effect of DTT on two ASIC subunits prominently expressed in sensory neurons, ASIC1b (a splice variant of ASIC1a) and ASIC3. DTT affected mASIC1b currents similarly to mASIC1a. The presence of DTT induced an eightfold increase in current amplitude ($826 \pm 135\%$ of pre-DTT control, $n = 8$, $P = 0.0004$; Fig. 7B). After the removal of DTT, currents remained potentiated ($230 \pm 34\%$, $n = 8$, $P = 0.007$). H⁺-gated currents from cells expressing mASIC3 were also impacted by DTT. The presence of DTT caused pH 6.0-activated mASIC3 currents to increase to $357 \pm 80\%$ of pre-DTT control ($n = 10$, $P = 0.009$; Fig. 7C). After removal of DTT, these currents remained elevated ($288 \pm 68\%$, $n = 10$, $P = 0.017$). Therefore, DTT potentiated the peak current amplitude of all ASICs examined.

Metal chelator TPEN mimics the transient effect of DTT on hASIC1a. Sulfhydryl compounds display two chemical properties that impact ion channel function. They modify the

Fig. 5. DTT and glutathione potentiate H⁺-gated currents in hippocampal neurons. *A*: representative traces show the potentiation of H⁺-gated currents activated at pH 6.0 by 1 mM DTT (4 min). *B*: time course of H⁺-gated currents at pH 6.0 in hippocampal neurons in the presence of DTT and following DTT removal (solid circles) and in mock-treated neurons (open circles). Note the rundown of H⁺-gated currents in mock-treated neurons. Peak current amplitudes were normalized to that of the pre-DTT or prevehicle control (100%, arrow). $**P < 0.01$ and $***P < 0.001$ compared with mock-treated control (unpaired *t*-test). *C*: effect of DTT on desensitization of H⁺-gated currents. $*P < 0.05$ (paired *t*-test). *D*: representative trace of the effect of 100 μ M glutathione on cultured hippocampal neurons (4-min glutathione incubation and 1-min washout). *E*: quantification of transient and long-lasting effects of 1 mM DTT and 100 μ M glutathione on H⁺-gated currents in hippocampal neurons. PreTx, pretreatment control. $**P < 0.01$ and $***P < 0.001$ compared with mock-treated group (unpaired *t*-test); $\#P < 0.05$, transient vs. long-lasting effects (paired *t*-test). Transient effect was measured during DTT or glutathione incubation (4 min), and the long-lasting effect was measured after washout (1 min). *F*: representative trace of the effect of DTT on H⁺-gated currents from ASIC2 knockout (KO) neurons. *G*: quantification of the effect on pH 6.5-induced current amplitude. $*P < 0.05$ and $**P < 0.01$ compared with pre-DTT control; $\#P < 0.05$, transient vs. long-lasting effects (paired *t*-test). *H*: effect of DTT on desensitization of H⁺-gated currents in ASIC2 knockout neurons. $**P < 0.01$ compared with pre-DTT control (paired *t*-test).

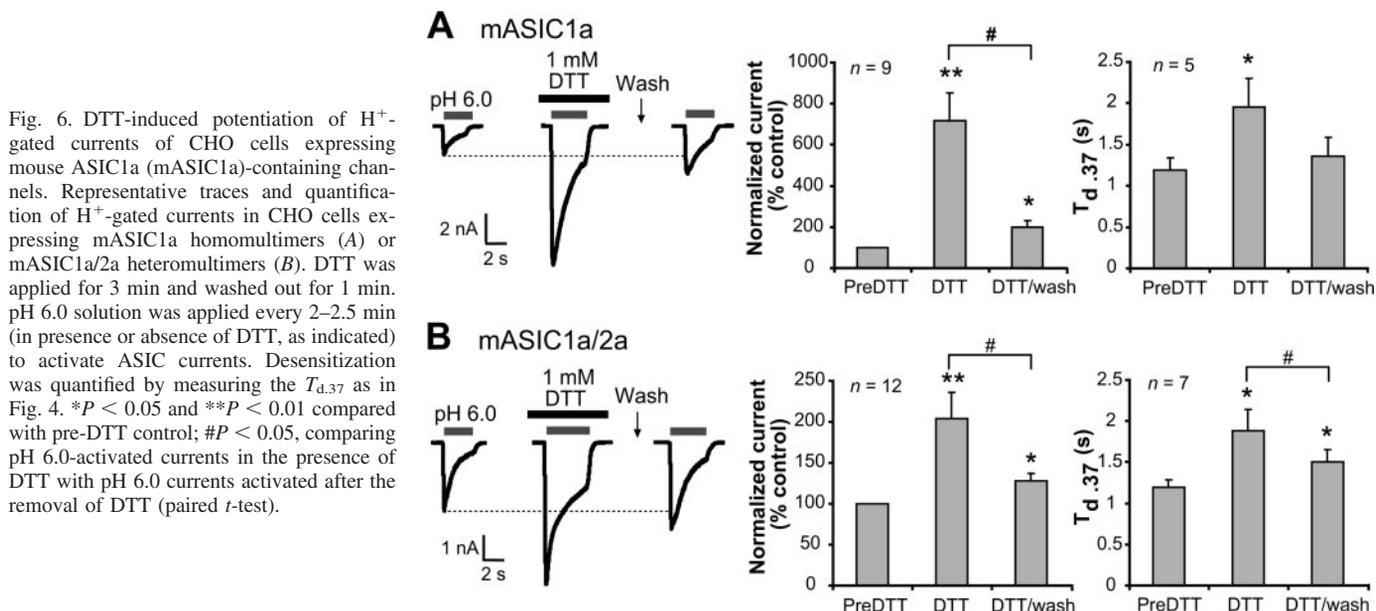


Fig. 6. DTT-induced potentiation of H⁺-gated currents of CHO cells expressing mouse ASIC1a (mASIC1a)-containing channels. Representative traces and quantification of H⁺-gated currents in CHO cells expressing mASIC1a homomultimers (A) or mASIC1a/2a heteromultimers (B). DTT was applied for 3 min and washed out for 1 min. pH 6.0 solution was applied every 2–2.5 min (in presence or absence of DTT, as indicated) to activate ASIC currents. Desensitization was quantified by measuring the $T_{d.37}$ as in Fig. 4. * $P < 0.05$ and ** $P < 0.01$ compared with pre-DTT control; # $P < 0.05$, comparing pH 6.0-activated currents in the presence of DTT with pH 6.0 currents activated after the removal of DTT (paired *t*-test).

structure and function of proteins by reducing amino acid residues and disulfide bonds. They also chelate transition metal ions such as Cu²⁺, Fe²⁺, Mn²⁺, Ni²⁺, and Zn²⁺. ASIC1a is inhibited by extracellular Zn²⁺ in the nanomolar range (17). Zinc decreases the apparent sensitivity of ASIC1a for protons, and ambient Zn²⁺ concentration in the extracellular solution is enough to tonically inhibit ASIC1a (17). We hypothesized that the acute potentiation of ASIC1a that is observed during DTT incubation and disappears after washing was due to Zn²⁺ chelation. To test this hypothesis, we used TPEN, a compound that chelates transition metal ions including zinc (Fig. 8). In the presence of 10 μM TPEN, peak H⁺-gated current amplitude of hASIC1a increased (Fig. 8A). The potentiation of hASIC1a current amplitude in the presence of either TPEN or DTT was remarkably similar (198 ± 18% for TPEN, 201 ± 13% for DTT, $n = 14$, $P = 0.99$; Fig. 8A). Like DTT, TPEN also slowed the desensitization of hASIC1a, and the $T_{d.37}$ in the presence of TPEN was not significantly different from the $T_{d.37}$ in the presence of DTT (4.25 ± 0.38 for DTT, 4.44 ± 0.23 for TPEN, $n = 5$, $P = 0.50$; Fig. 8B). However, TPEN-induced potentiation did diverge from DTT potentiation in one respect. Potentiation disappeared completely after the removal of TPEN, whereas currents remained potentiated following the removal of DTT (107 ± 5% and 150 ± 12% after the washout of TPEN and DTT, respectively, $P = 0.013$, $n = 7$; Fig. 8A). These results are consistent with the proposed mechanism of action of these two compounds. While TPEN simply chelates transition metals, DTT can both chelate transition metals and covalently modify amino acids residues to induce long-lasting effects on channel function.

To determine whether the effects of TPEN and DTT are additive, we studied the consequences of coapplication of both compounds. We found that application of DTT in the presence of TPEN caused no additional increase in hASIC1a current activated by pH 6.5 solutions (231 ± 34% for TPEN, 246 ± 22% for TPEN + DTT, $n = 6$, $P = 0.49$; Fig. 8C). Similar results were attained when the activating pH was 6.7 (265 ± 55% for TPEN, 263 ± 49% for TPEN + DTT, $n = 5$, $P =$

0.82; Fig. 8C). These results indicate that potentiation of hASIC1a currents in the presence of DTT is due to metal chelation.

Mutation K133R attenuates both DTT- and TPEN-induced potentiation of hASIC1a. If potentiation of hASIC1a currents in the presence of DTT is due to zinc chelation, then mutant channels insensitive to extracellular zinc should not be acutely affected by DTT. In mASIC1a, a substitution of lysine 133 to an arginine eliminates high-affinity Zn²⁺ inhibition (17). We made this substitution in hASIC1a (K113R), transfected it into CHO cells, and analyzed the effects of TPEN and DTT on H⁺-gated currents (Fig. 9). Similar to previous reports on mASIC1a with this substitution, the pH dose-response curve of K133R was similar to that of hASIC1a (data not shown). TPEN-induced potentiation of H⁺-gated currents from K133R was dramatically reduced compared with wildtype hASIC1a (138 ± 6% for K133R and 202 ± 23% for hASIC1a, $P = 0.019$; Fig. 9, A–C). Thus K133R shows significantly reduced tonic inhibition by zinc, but some transition metal inhibition still remains (138 ± 6%, $n = 9$, $P = 0.0004$). DTT-induced potentiation was also reduced in this mutant channel (Fig. 9B). In the presence of DTT, the increase in H⁺-gated currents of K133R was only 131 ± 10% compared with hASIC1a potentiation of 204 ± 15% ($n = 9$, $P = 0.0007$; Fig. 9C). After the removal of DTT, H⁺-gated currents of K133R were only 113 ± 4% of the pre-DTT control, whereas hASIC1a was 151 ± 10% of pre-DTT control ($P = 0.023$; Fig. 9C). These results indicate that the long-lasting effect of DTT, which we ascribe to redox modulation of the channel, is also compromised in K133R. Thus both TPEN- and DTT-induced potentiation is attenuated in the K133R mutant of hASIC1a. These results indicate that lysine 133 affects both TPEN and DTT potentiation and suggests that these compounds have similar mechanisms of action.

ASIC1b and ASIC3 are potentiated by the metal chelator TPEN. Our studies suggest that the presence of DTT potentiates ASIC1a by chelating transition metal ions that tonically inhibit H⁺-gated currents (Figs. 8–9). Consistent with this

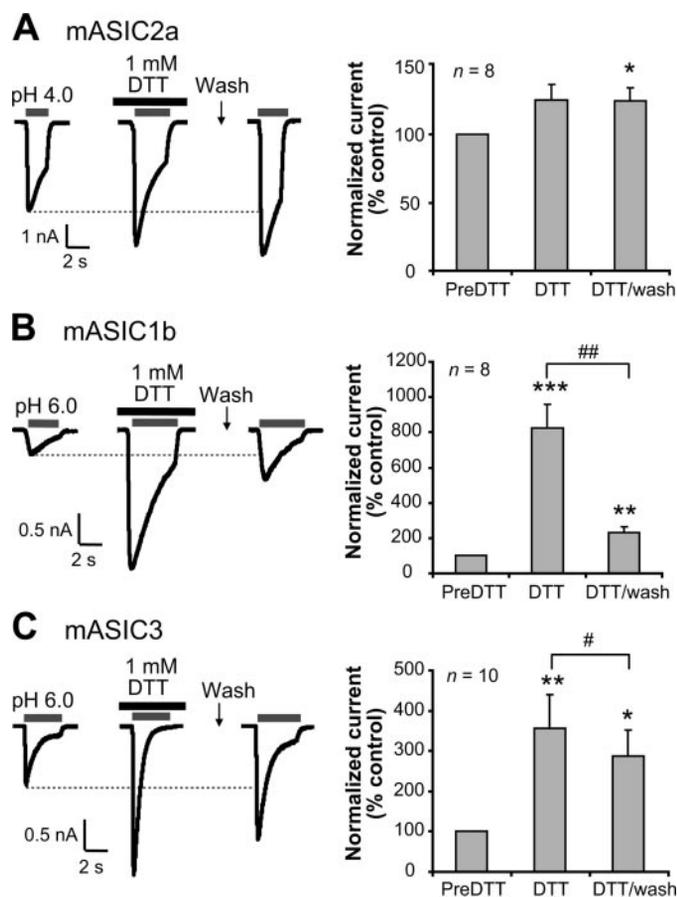


Fig. 7. DTT potentiates mASIC2a, mASIC1b, and mASIC3 H^+ -gated currents. Representative trace and quantification of the effect of DTT on mASIC2a (A), mASIC1b (B), and mASIC3 (C) expressed in CHO cells. ASIC currents were activated by pH 4.0 or 6.0 solutions every 2 min. DTT (1 mM) was applied for 3 min. During this 3-min time period, ASIC currents were evoked by application of pH 4.0 or 6.0 solutions in the presence of DTT. DTT was removed by exchanging the bath solution and superfusing the cell with DTT-free solution for 1 min. pH 4.0 or 6.0 solution (in the absence of DTT) was then applied. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with pre-DTT control; # $P < 0.05$ and ## $P < 0.01$, comparing pH 6.0 amplitude in the presence of DTT and after DTT removal (paired t -test).

idea, zinc inhibits ASIC1a currents at concentrations normally present in extracellular solutions (17), and our results indicate that the metal chelator TPEN mimics DTT-induced potentiation (Fig. 8, A–B). The effects of both TPEN and DTT are not additive (Fig. 8C), suggesting that they act through a common mechanism. However, DTT potentiates ASIC1b and ASIC3 (Fig. 7, B–C), which are not reported to be sensitive to extracellular zinc (17). To examine whether these channels are affected by metals and whether transient potentiation by DTT is due to metal chelation, we expressed mASIC1a, mASIC1b, and mASIC3 in CHO cells and then analyzed the effect of TPEN and DTT on H^+ -gated currents (Fig. 10). Like hASIC1a, 10 μ M TPEN potentiated mASIC1a current, and the application of DTT in the presence of TPEN did not result in an additional increase in mASIC1a current ($581 \pm 122\%$ for TPEN, $579 \pm 134\%$ for TPEN + DTT, $n = 5$, $P = 0.96$; Fig. 10A). mASIC1b was also enhanced by TPEN in a concentration-dependent manner ($593 \pm 149\%$, $n = 6$, $P = 0.02$ for 10 μ M TPEN; $882 \pm 213\%$, $n = 4$, $P = 0.03$ for 100 μ M TPEN; Fig. 10B). DTT further increased ASIC1b currents in the

presence of 10 μ M TPEN but not 100 μ M TPEN ($n = 5$, $P = 0.008$ for 10 μ M TPEN, and $n = 4$, $P = 0.84$ for 100 μ M TPEN compared with TPEN alone; Fig. 10B). These results suggest that mASIC1a and mASIC1b differ in their sensitivity to metal chelators. TPEN (10 μ M) also increased ASIC3 currents ($164 \pm 14\%$, $n = 8$, $P = 0.003$; Fig. 10C). When coapplied with 10 μ M TPEN, there was no additive enhancement of ASIC3 currents ($n = 4$, $P = 0.18$ compared with TPEN alone; Fig. 10C). These results suggest that, like ASIC1a, the chelation of transition metal ions may underlie potentiation of ASIC1b and ASIC3 by DTT.

DISCUSSION

The ASICs are important for normal behavior and sensory transduction (13, 25, 31, 35, 36, 50–52). In addition, calcium-permeable ASIC1a contributes to neuronal death following ischemia in the brain (55). Therefore, understanding the factors that influence ASIC1a activity may lend insight into the modulators that control cell death following ischemia and stroke. In this study, we show that the activity of ASICs, including hASIC1a, is enhanced by sulfhydryl compounds. Since the extracellular concentration of sulfhydryls can change in physiological and pathological states (4, 29, 38, 57), the modulation of ASICs by these compounds may have a strong impact on ASIC function in the central and peripheral nervous system.

Similar to previous studies in mouse sensory neurons and cortical neurons (5, 15), we found that sulfhydryl agents enhanced ASIC currents. DTT shifts the pH dose-response curve of both human and mouse ASIC1a such that less acidic pHs are required to activate H^+ -gated currents (15). Thus sulfhydryl-induced potentiation of ASIC1a is conserved between species, although there are differences in DTT modulation of human and mouse ASICs. We observed an extremely robust (sevenfold) increase of mASIC1a currents in the presence of DTT, whereas hASIC1a was potentiated only twofold. This difference could be ascribed to the different pH solutions used to activate currents (pH 6.5 for hASIC1a and pH 6.0 for mASIC1a). However, at no place on the hASIC1a pH dose-response curve did we observe a sevenfold potentiation of current by DTT. Human and mouse ASIC1a channels are highly conserved, with only 11 amino acid differences. Our results suggest that at least one of these amino acid differences affects how sulfhydryl reagents impact channel characteristics.

We also observed differences in potentiation between H^+ -gated currents of mouse hippocampal neurons and heterologously expressed mASICs. In particular, DTT-induced potentiation of H^+ -gated currents in ASIC2 knockout neurons (which have predominantly mASIC1a-like currents) was much smaller than that of mASIC1a expressed in CHO cells. This difference could be due to the state of neuronal ASICs (some may already be reduced or free of metal), the presence of other ASIC subunits, or neuron-specific modification of ASIC1a.

Sulfhydryl compounds modulate many ion channels by reducing disulfide bonds and chelating transition metal ions (30, 53). For example, DTT produces an increase in the *N*-methyl-D-aspartate (NMDA) receptor current that persists even after removal of DTT. It is thought that this effect is produced by reduction of disulfide bonds between two cysteine residues (C744 and C798) in the NR1 subunit (42). DTT also increases NMDA receptor activity by chelating transition met-

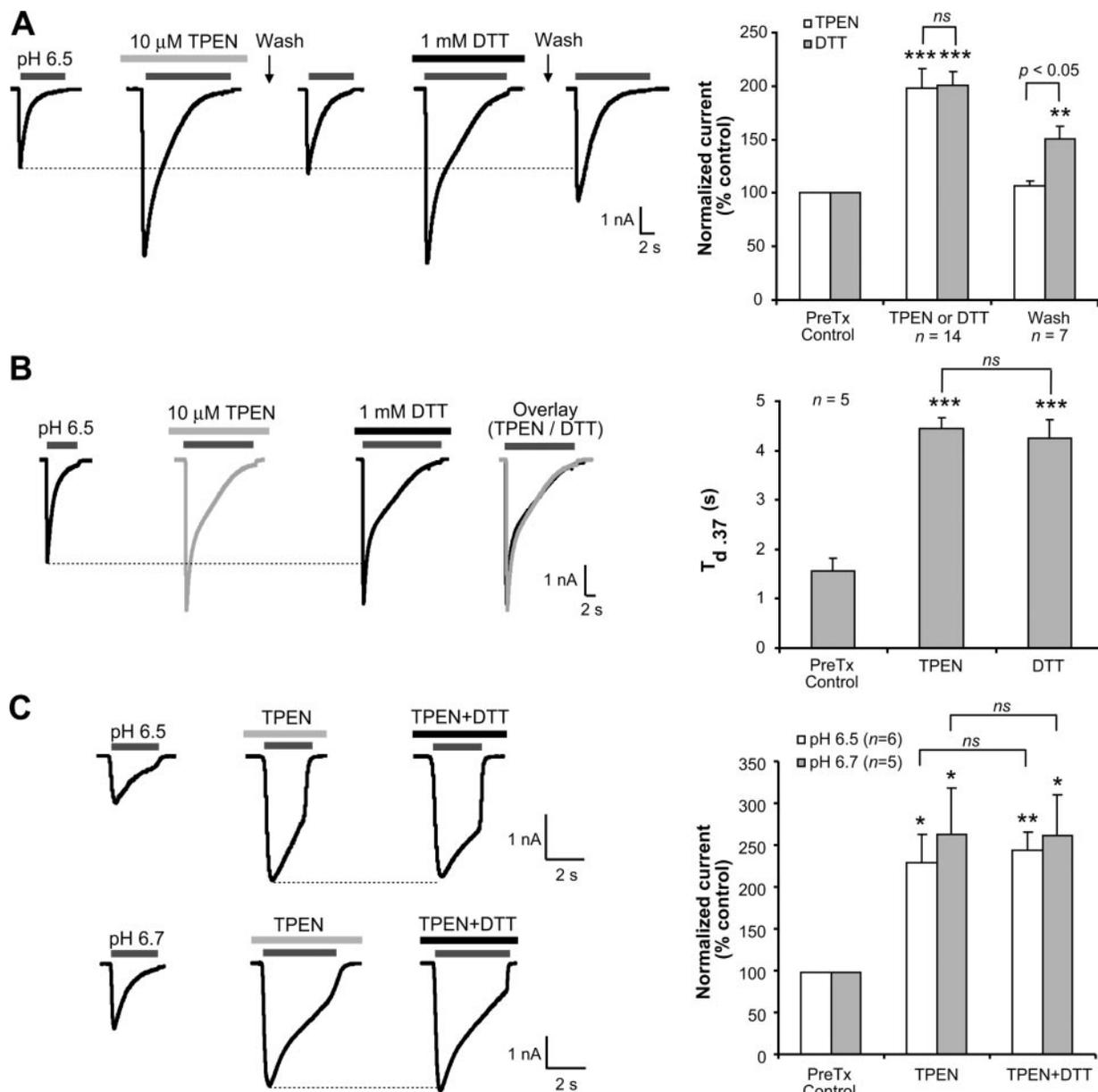


Fig. 8. The metal chelator TPEN mimics the transient effect of DTT. *A*: representative trace of hASIC1a H^+ -gated currents in the presence of TPEN (10 μ M) or DTT (1 mM). Quantification of the peak current amplitude of pH 6.5-activated currents in the presence of TPEN or DTT and after washout. $**P < 0.01$ and $***P < 0.001$ (paired *t*-test). There was no significant difference (ns) in the acute potentiation by TPEN and by DTT. *B*: representative trace and quantification of the change in desensitization when either TPEN or DTT is applied. $***P < 0.001$ (paired *t*-test). *C*: representative trace of the effect of applying both TPEN and DTT concurrently and quantification of the potentiation of hASIC1a by TPEN or TPEN + DTT. Peak amplitude values were normalized to that of the pretreatment control (PreTx, 100%). There was no significant difference (ns) in the potentiation by TPEN and by TPEN + DTT. $*P < 0.05$ and $***P < 0.01$ (paired *t*-test).

als and relieving tonic Zn^{2+} inhibition (33). Our data suggest that DTT affects ASIC1a in a similar manner. In the presence of DTT or glutathione, the peak current amplitude of hASIC1a currents is potentiated, and desensitization is slowed. This effect is mimicked by the addition of the metal chelator TPEN, and the addition of DTT with TPEN does not increase currents further. Furthermore, the increase in currents induced by DTT was significantly attenuated in a mutant channel (K133R) that is less sensitive to extracellular zinc. Together, these results indicate that DTT potentiates hASIC1a by chelating transition metals. Sulfhydryls also potentiate ASIC1b and ASIC3 currents. Like ASIC1a, ASIC1b and ASIC3 currents were not

further potentiated by DTT in the presence of TPEN, suggesting that the presence of DTT increases currents by chelating transition metal ions that tonically inhibit ASIC1b and ASIC3.

hASIC1a currents remain potentiated following DTT incubation and removal. This long-lasting potentiation is reversed by the addition of DTNB, which covalently modifies free sulfhydryl groups. Additionally, this long-lasting effect is not induced by treatment with TPEN alone. These results suggest that DTT impacts peak current amplitude of hASIC1a by reducing amino acid residues within the ASIC1a protein as well as metal chelation. The fact that all ASICs examined showed long-lasting potentiation after the removal of DTT

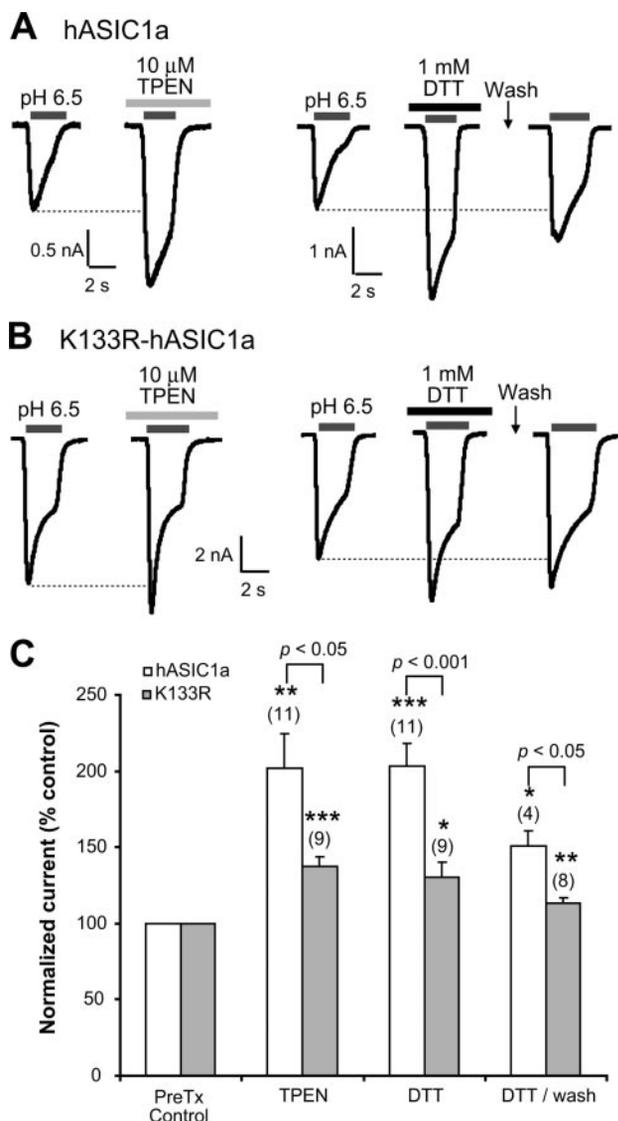


Fig. 9. Lysine 133 mutation reduces both TPEN- and DTT-induced potentiation of hASIC1a. Representative traces of the potentiation of hASIC1a (A) and K133R-hASIC1a (B) by TPEN (10 μ M, 1 min) and DTT (1 mM, 3 min). H^+ -gated currents at pH 6.5 were recorded before, during, and after incubation of TPEN or DTT. C: quantification of the effect of TPEN and DTT on hASIC1a and K133R. Nos. in parentheses represent nos. of cells examined. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with pretreatment control (paired t -test).

suggests that the residue(s) undergoing redox modulation is conserved among ASIC subunits. Since glutathione is not readily membrane permeable, residues within the extracellular domain are likely targets for sulfhydryl-induced reduction. Free and disulfide-bonded cysteine residues are major sites for redox modulation by oxidizing and reducing agents (30). ASICs have 14 conserved cysteine residues within their extracellular domain (27). These residues are thought to form disulfide bonds that impact the tertiary structure. Conserved cysteine residues are essential for cell surface expression of the related DEG/ENaC channel (20). The role of the conserved cysteine residues in the extracellular domain of ASICs has not yet been reported. Our results suggest that modification of these cysteine residues in ASICs impacts channel function.

Although all ASICs showed long-lasting potentiation after DTT incubation, only ASIC1a, -1b, and -3 displayed increased potentiation in the presence of DTT compared with the potentiation following DTT removal. These three channels were also potentiated by TPEN, suggesting that they are tonically inhibited by basal concentrations of transition metals. Either TPEN or DTT chelates metals and frees the ion channel from metal inhibition. It is known that ASIC1a channels are inhibited by extracellular zinc at concentrations that are typically present in

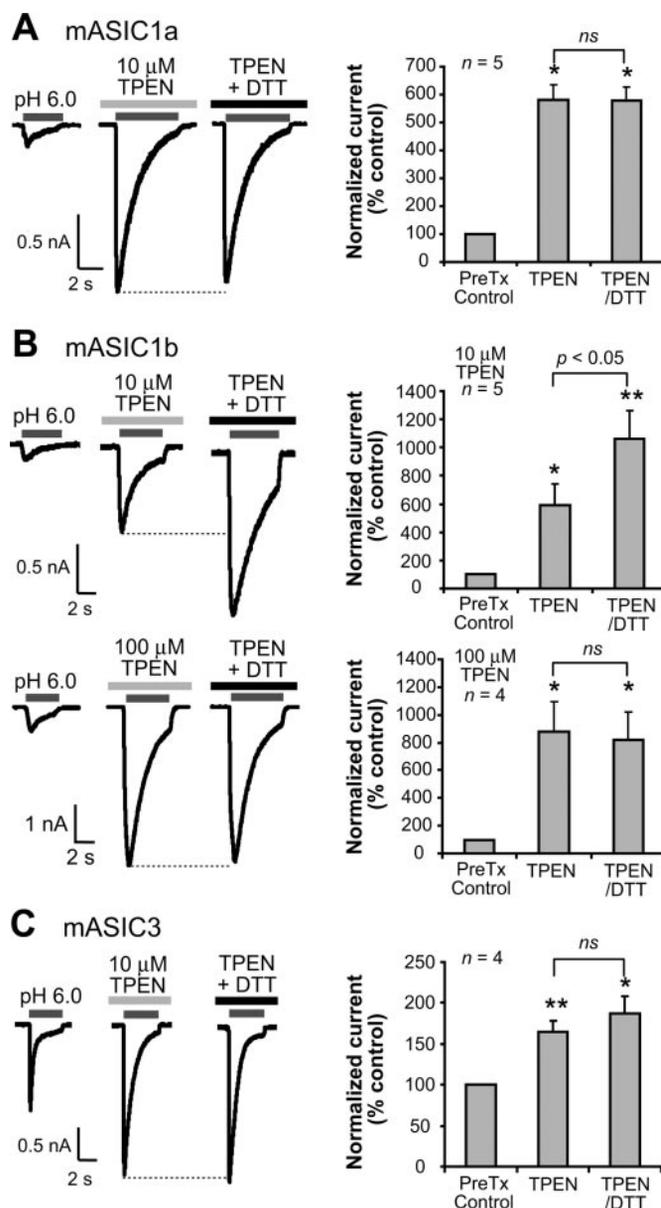


Fig. 10. Effect of TPEN and DTT on mASIC1a, mASIC1b, and mASIC3. Representative trace and quantification of effect of TPEN and DTT addition on mASIC1a (A), mASIC1b (B), and mASIC3 (C) expressed in CHO cells. H^+ -gated currents were activated by pH 6.0 solution in the presence of the indicated compounds. TPEN (10 or 100 μ M) was applied for 1 min at pH 7.4 before activation of H^+ -gated currents. When DTT and TPEN were both present during the acid stimulus (TPEN + DTT), TPEN was applied for 1 min at pH 7.4, and then DTT (1 mM) was added in the continued presence of TPEN at pH 7.4 for an additional 1 min before acid-dependent activation. * $P < 0.05$ and ** $P < 0.01$ compared with pretreatment control values (no DTT or TPEN) (paired t -test).

experimental solutions (15). Like DTT, zinc chelation with TPEN is known increase the apparent proton sensitivity of ASIC1a (17). The removal of zinc, however, has not been reported to impact channel desensitization, and we observe robust changes in desensitization of hASIC1a with the addition of TPEN or DTT. Recent work has shown that lead, cadmium, nickel, and copper inhibit ASIC1a currents (40, 48, 49). Furthermore, we found that TPEN potentiated ASIC1b and ASIC3 channels reported to be insensitive to zinc. It is well known that TPEN, DTT, and glutathione chelate other transition metal ions as well as or even better than zinc. For example, the stability constants ($\log_{10}K_C$) for the chelation by TPEN are 20.6 for Cu^{2+} , 14.6 for Fe^{2+} , 10.3 for Mn^{2+} , 18.0 for Ni^{2+} , and 18.0 for Zn^{2+} (39). For the chelation of Cd^{2+} , Cu^{2+} , Ni^{2+} , Pb^{2+} , and Zn^{2+} , $\log_{10}K_C$ (DTT) = 14.6, 15.3, 10.7, 13.7, and 11.1, respectively, and $\log_{10}K_C$ (glutathione) = 10.2, 15.5, 10.4, 10.6, and 8.0, respectively (28, 39). Our data suggest that ASICs may be sensitive to one of these metals in addition to zinc. Further experiments are necessary to determine the identity of the metal responsible for the basal inhibition in our experiments. We cannot exclude the possibility that our solutions contain small amounts of other transition metals that inhibit ASIC currents.

Sulfhydryl modification of ASICs has been described previously (5, 15). Although both studies reported potentiation of H^+ -gated currents by DTT, the specific changes in H^+ -gated current characteristics and the proposed mechanisms are different. For example, Chu et al. (15) reported that DTT potentiates H^+ -gated currents twofold, whereas Andrey et al. (5) observed only 46% potentiation. Chu et al. (15) focused on mASIC1a and H^+ -gated currents in neurons from the cortex. Andrey et al. (5) worked in sensory neurons, which express ASIC1b and ASIC3 in addition to ASIC1a and ASIC2. Our results may explain the discrepancies between these two studies. We found that mASIC1b, mASIC2a, and mASIC3 were all potentiated by DTT. The results obtained from sensory neurons may represent DTT modification of heteromultimeric channels composed of these other subunits, which may have very different levels of potentiation compared with mASIC1a. Chu et al. (15) also found that DTT shifts the pH dose-response curve of mASIC1a-containing channels and does not impact peak current amplitude when the channel is activated by a maximal acidic stimulus (15). However, Andrey et al. (5) found that DTT did not affect the pH dose-response and increased the peak current amplitude of pH 4.5-activated currents in sensory neurons (5). DTT-induced potentiation of H^+ -gated currents in sensory neurons may be due to potentiation of ASIC1b-, ASIC2a-, or ASIC3-containing subunits that may be impacted differently from ASIC1a and show a different pH sensitivity of potentiation.

Our results also diverge from previous reports. We find that all ASICs tested were potentiated by DTT. However, Chu et al. (15) reported that only ASIC1a-containing channels are affected by DTT. In addition, we report that the largest potentiation by DTT is due to metal chelation. Chu et al. (15) reported that the effect of DTT was not due to chelation of transition metals. The exact reason for these discrepancies is unclear, although we can offer several explanations. First, the majority of our studies focused specifically on ASIC potentiation in the presence of sulfhydryls. Second, we used the metal chelator TPEN, which has a very high affinity for metals. Previous

publications used tricine, a relatively weak chelator of transition metals (1, 15). For the chelation of Cd^{2+} , Ni^{2+} , Pb^{2+} , and Zn^{2+} , the stability constant $\log_{10}K_C$ (tricine) = 4.4, 5.5, 4.3, and 5.6, respectively (1). We observed that DTT further increased hASIC1a currents in the presence of 10 mM tricine ($121 \pm 5\%$, $n = 5$, $P = 0.015$) but not 10 μM TPEN. This suggests that the use of TPEN rather than tricine is responsible, at least in part, for the discrepant results (15). One final possibility is that our solutions may contain a different basal concentration of metals. As stated before, our experiments did not distinguish which transition metal was inhibiting ASIC activity. For example, the concentration of contaminating zinc is usually in the nanomolar range for experimental solutions (3). We found that our solutions contained 150 nM zinc as well as nanomolar concentrations of chromium, copper, and nickel. It is likely that additional metals inhibit ASIC activity. It is also likely that the types and concentration of metals will vary in solutions among different laboratories. Thus our solutions may have contained a basal concentration of a metal that inhibits ASIC activity that was not present within the solutions of other investigators.

The concentration of extracellular sulfhydryls is altered in both pathological and physiological conditions. The extracellular concentration of endogenous sulfhydryl compounds increases in the ischemic brain (4, 29, 38). Glutathione is abundant in neurons and glial cells and is released during ischemia from cells with damaged membranes. In addition, oxygen/glucose deprivation opens neuronal gap junction hemichannels, resulting in the loss of ionic homeostasis and the efflux of cytosolic molecules <1 kDa in size (43). This raises the possibility that reduced glutathione may be released from neurons through hemichannels activated during ischemia, even when cell membranes remain intact. It is known that the extracellular concentration of glutathione and cysteine can increase to 1 and 5 μM , respectively, in rat models of focal ischemia (29). This increase in sulfhydryls has been shown to impact the activity of multiple ion channels involved in ischemia-induced neuronal death. Interestingly, glutathione potentiates both NMDA receptors and ASICs, two channels known to enhance excitotoxic and acidotoxic neuronal death following stroke and ischemia. Sulfhydryl modulation of ASICs could have profound effects on neuronal death following stroke.

Neuronal activity also induces the release of endogenous sulfhydryl compounds, which in turn regulate ion channels involved in synaptic transmission and plasticity (22, 57). Low levels of glutathione have previously been linked to impaired synaptic plasticity (2, 18, 41). ASIC1a localizes to synapses, and ASIC1 knockout animals show impaired performance in a number of behavioral tests of learning and memory (50, 51). ASIC1 also plays a prominent role in fear-related behaviors (52). Interestingly, the enzyme that maintains the levels of reduced glutathione in the brain has also been linked to fear- and anxiety-related behaviors in mice (24). Thus the potentiation of ASICs by sulfhydryl compounds may regulate synaptic plasticity, learning and memory, and possibly fear-related behaviors.

ACKNOWLEDGMENTS

We thank Jack Enyeart and Kirk Mykityn for comments on previous versions of the manuscript and for many discussions. We thank Michael

Welsh, Margaret Price, and John Wemmie (Univ. of Iowa, Iowa City, IA) for providing the ASIC clones and the ASIC2 knockout mice.

GRANTS

This work was supported by Grant IBN-0416920 from the National Science Foundation (to C. C. Askwith).

REFERENCES

- Ahmed IT. Formation constants of ternary complexes involving some metal ions, tricine, dicarboxylic amino acids, as well as *N*-(2-acetamido)iminodiacetic acid and 3-amino-5-mercapto-1,2,4-triazole. *J Chem Eng Data* 48: 272–276, 2003.
- Almaguer-Melian W, Cruz-Aguado R, Bergado JA. Synaptic plasticity is impaired in rats with a low glutathione content. *Synapse* 38: 369–374, 2000.
- Amar M, Perin-Dureau F, Neyton J. High-affinity Zn block in recombinant *N*-methyl-D-aspartate receptors with cysteine substitutions at the Q/R/N site. *Biophys J* 81: 107–116, 2001.
- Andine P, Orwar O, Jacobson I, Sandberg M, Hagberg H. Extracellular acidic sulfur-containing amino acids and gamma-glutamyl peptides in global ischemia: postischemic recovery of neuronal activity is paralleled by a tetrodotoxin-sensitive increase in cysteine sulfinate in the CA1 of the rat hippocampus. *J Neurochem* 57: 230–236, 1991.
- Andrey F, Tsintsadze T, Volkova T, Lozovaya N, Krishtal O. Acid sensing ionic channels: modulation by redox reagents. *Biochim Biophys Acta* 1745: 1–6, 2005.
- Askwith CC, Cheng C, Ikuma M, Benson C, Price MP, Welsh MJ. Neuropeptide FF and FMRFamide potentiate acid-evoked currents from sensory neurons and proton-gated DEG/ENaC channels. *Neuron* 26: 133–141, 2000.
- Askwith CC, Wemmie JA, Price MP, Rokhlina T, Welsh MJ. Acid-sensing ion channel 2 (ASIC2) modulates ASIC1 H⁺-activated currents in hippocampal neurons. *J Biol Chem* 279: 18296–18305, 2004.
- Baron A, Schaefer L, Lingueglia E, Champigny G, Lazdunski M. Zn²⁺ and H⁺ are coactivators of acid-sensing ion channels. *J Biol Chem* 276: 35361–35367, 2001.
- Bassilana F, Champigny G, Waldmann R, de Weille JR, Heurteaux C, Lazdunski M. The acid-sensitive ionic channel subunit ASIC and the mammalian degenerin MDEG form a heteromultimeric H⁺-gated Na⁺ channel with novel properties. *J Biol Chem* 272: 28819–28822, 1997.
- Benson CJ, Xie J, Wemmie JA, Price MP, Henss JM, Welsh MJ, Snyder PM. Heteromultimers of DEG/ENaC subunits form H⁺-gated channels in mouse sensory neurons. *Proc Natl Acad Sci USA* 99: 2338–2343, 2002.
- Bubien JK, Ji HL, Gillespie GY, Fuller CM, Markert JM, Mapstone TB, Benos DJ. Cation selectivity and inhibition of malignant glioma Na⁺ channels by Psalmitoxin 1. *Am J Physiol Cell Physiol* 287: C1282–C1291, 2004.
- Catarsi S, Babinski K, Seguela P. Selective modulation of heteromeric ASIC proton-gated channels by neuropeptide FF. *Neuropharmacology* 41: 592–600, 2001.
- Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ. A role for ASIC3 in the modulation of high-intensity pain stimuli. *Proc Natl Acad Sci USA* 99: 8992–8997, 2002.
- Chen X, Paukert M, Kadurin I, Pusch M, Grunder S. Strong modulation by RFamide neuropeptides of the ASIC1b/3 heteromer in competition with extracellular calcium. *Neuropharmacology* 50: 964–974, 2006.
- Chu XP, Close N, Saugstad JA, Xiong ZG. ASIC1a-specific modulation of acid-sensing ion channels in mouse cortical neurons by redox reagents. *J Neurosci* 26: 5329–5339, 2006.
- Chu XP, Miesch J, Johnson M, Root L, Zhu XM, Chen D, Simon RP, Xiong ZG. Proton-gated channels in PC12 cells. *J Neurophysiol* 87: 2555–2561, 2002.
- Chu XP, Wemmie JA, Wang WZ, Zhu XM, Saugstad JA, Price MP, Simon RP, Xiong ZG. Subunit-dependent high-affinity zinc inhibition of acid-sensing ion channels. *J Neurosci* 24: 8678–8689, 2004.
- Cruz-Aguado R, Almaguer-Melian W, Diaz CM, Lorigados L, Bergado J. Behavioral and biochemical effects of glutathione depletion in the rat brain. *Brain Res Bull* 55: 327–333, 2001.
- Deval E, Baron A, Lingueglia E, Mazarguil H, Zajac JM, Lazdunski M. Effects of neuropeptide SF and related peptides on acid sensing ion channel 3 and sensory neuron excitability. *Neuropharmacology* 44: 662–671, 2003.
- Firsov D, Robert-Nicoud M, Gruender S, Schild L, Rossier BC. Mutational analysis of cysteine-rich domains of the epithelium sodium channel (ENaC). Identification of cysteines essential for channel expression at the cell surface. *J Biol Chem* 274: 2743–2749, 1999.
- Gao J, Wu LJ, Xu L, Xu TL. Properties of the proton-evoked currents and their modulation by Ca²⁺ and Zn²⁺ in the acutely dissociated hippocampus CA1 neurons. *Brain Res* 1017: 197–207, 2004.
- Gozlan H, Ben-Ari Y. NMDA receptor redox sites: are they targets for selective neuronal protection? *Trends Pharmacol Sci* 16: 368–374, 1995.
- Hesselager M, Timmermann DB, Ahring PK. pH dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. *J Biol Chem* 279: 11006–11015, 2004.
- Hovatta I, Tennant RS, Helton R, Marr RA, Singer O, Redwine JM, Ellison JA, Schadt EE, Verma IM, Lockhart DJ, Barlow C. Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. *Nature* 438: 662–666, 2005.
- Jones NG, Slater R, Cadiou H, McNaughton P, McMahon SB. Acid-induced pain and its modulation in humans. *J Neurosci* 24: 10974–10979, 2004.
- Jones RC 3rd, Xu L, Gebhart GF. The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J Neurosci* 25: 10981–10989, 2005.
- Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. *Physiol Rev* 82: 735–767, 2002.
- Krezel A, Lesniak W, Jezowska-Bojczek M, Mlynarz P, Brasun J, Kozlowski H, Bal W. Coordination of heavy metals by dithiothreitol, a commonly used thiol group protectant. *J Inorg Biochem* 84: 77–88, 2001.
- Landolt H, Lutz TW, Langemann H, Stauble D, Mendelowitsch A, Gratzl O, Honegger CG. Extracellular antioxidants and amino acids in the cortex of the rat: monitoring by microdialysis of early ischemic changes. *J Cereb Blood Flow Metab* 12: 96–102, 1992.
- Lipton SA, Choi YB, Takahashi H, Zhang D, Li W, Godzik A, Bankston LA. Cysteine regulation of protein function—as exemplified by NMDA-receptor modulation. *Trends Neurosci* 25: 474–480, 2002.
- Mogil JS, Breese NM, Witt MF, Ritchie J, Rainville ML, Ase A, Abbadi N, Stucky CL, Seguela P. Transgenic expression of a dominant-negative ASIC3 subunit leads to increased sensitivity to mechanical and inflammatory stimuli. *J Neurosci* 25: 9893–9901, 2005.
- Ostrovskaya O, Moroz L, Krishtal O. Modulatory action of RFamide-related peptides on acid-sensing ion channels is pH dependent: the role of arginine. *J Neurochem* 91: 252–255, 2004.
- Paoletti P, Ascher P, Neyton J. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J Neurosci* 17: 5711–5725, 1997.
- Poirot O, Berta T, Decosterd I, Kellenberger S. Distinct ASIC currents are expressed in rat putative nociceptors and are modulated by nerve injury. *J Physiol* 576: 215–234, 2006.
- Price MP, Lewin GR, McIlwraith SL, Cheng C, Xie J, Heppenstall PA, Stucky CL, Mannsfeldt AG, Brennan TJ, Drummond HA, Qiao J, Benson CJ, Tarr DE, Hrstka RF, Yang B, Williamson RA, Welsh MJ. The mammalian sodium channel BNC1 is required for normal touch sensation. *Nature* 407: 1007–1011, 2000.
- Price MP, McIlwraith SL, Xie J, Cheng C, Qiao J, Tarr DE, Sluka KA, Brennan TJ, Lewin GR, Welsh MJ. The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. *Neuron* 32: 1071–1083, 2001.
- Siesjo BK. Acidosis and ischemic brain damage. *Neurochem Pathol* 9: 31–88, 1988.
- Slivka A, Cohen G. Brain ischemia markedly elevates levels of the neurotoxic amino acid, cysteine. *Brain Res* 608: 33–37, 1993.
- Smith RM, Martell AE. *Critical Stability Constants*. New York: Plenum, 1974.
- Staruschenko A, Dorofeeva NA, Bolshakov KV, Stockand JD. Subunit-dependent cadmium and nickel inhibition of acid-sensing ion channels. *J Neurobiol* 67: 97–107, 2006.
- Stellet P, Neijt HC, Cuenod M, Do KQ. Synaptic plasticity impairment and hypofunction of NMDA receptors induced by glutathione deficit: relevance to schizophrenia. *Neuroscience* 137: 807–819, 2006.
- Sullivan JM, Traynelis SF, Chen HS, Escobar W, Heinemann SF, Lipton SA. Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. *Neuron* 13: 929–936, 1994.

43. **Thompson RJ, Zhou N, MacVicar BA.** Ischemia opens neuronal gap junction hemichannels. *Science* 312: 924–927, 2006.
44. **Vila-Carriles WH, Kovacs GG, Jovov B, Zhou ZH, Pahwa AK, Colby G, Esimai O, Gillespie GY, Mapstone TB, Markert JM, Fuller CM, Bubien JK, Benos DJ.** Surface expression of ASIC2 inhibits the amiloride-sensitive current and migration of glioma cells. *J Biol Chem* 281: 19220–19232, 2006.
45. **Vukicevic M, Kellenberger S.** Modulatory effects of acid-sensing ion channels (ASICs) on action potential generation in hippocampal neurons. *Am J Physiol Cell Physiol* 287: C682–C690, 2004.
46. **Waldmann R.** Proton-gated cation channels-neuronal acid sensors in the central and peripheral nervous system. *Adv Exp Med Biol* 502: 293–304, 2001.
47. **Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M.** A proton-gated cation channel involved in acid-sensing. *Nature* 386: 173–177, 1997.
48. **Wang W, Duan B, Xu H, Xu L, Xu TL.** Calcium-permeable acid-sensing ion channel is a molecular target of the neurotoxic metal ion lead. *J Biol Chem* 281: 2497–2505, 2006.
49. **Wang W, Yu Y, Xu TL.** Modulation of acid-sensing ion channels by Cu^{2+} in cultured hypothalamic neurons of the rat. *Neuroscience* 145: 631–641, 2007.
50. **Wemmie JA, Askwith CC, Lamani E, Cassell MD, Freeman JH Jr, Welsh MJ.** Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. *J Neurosci* 23: 5496–5502, 2003.
51. **Wemmie JA, Chen J, Askwith CC, Hruska-Hageman AM, Price MP, Nolan BC, Yoder PG, Lamani E, Hoshi T, Freeman JH Jr, Welsh MJ.** The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* 34: 463–477, 2002.
52. **Wemmie JA, Coryell MW, Askwith CC, Lamani E, Leonard AS, Sigmund CD, Welsh MJ.** Overexpression of acid-sensing ion channel 1a in transgenic mice increases acquired fear-related behavior. *Proc Natl Acad Sci USA* 101: 3621–3626, 2004.
53. **Wilkins ME, Smart TG.** Redox modulation of GABAA receptors obscured by Zn^{2+} complexation. *Neuropharmacology* 43: 938–944, 2002.
54. **Xie J, Price MP, Wemmie JA, Askwith CC, Welsh MJ.** ASIC3 and ASIC1 mediate FMRFamide-related peptide enhancement of H^+ -gated currents in cultured dorsal root ganglion neurons. *J Neurophysiol* 89: 2459–2465, 2003.
55. **Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP.** Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 118: 687–698, 2004.
56. **Yermolaieva O, Leonard AS, Schnizler MK, Abboud FM, Welsh MJ.** Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. *Proc Natl Acad Sci USA* 101: 6752–6757, 2004.
57. **Zangerle L, Cuenod M, Winterhalter KH, Do KQ.** Screening of thiol compounds: depolarization-induced release of glutathione and cysteine from rat brain slices. *J Neurochem* 59: 181–189, 1992.

