Published in final edited form as:

Cell Calcium. 2009 April ; 45(4): 319–325. doi:10.1016/j.ceca.2008.12.002.

Native and recombinant ASIC1a receptors conduct negligible Ca^{2+} entry

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Abstract

Acid Sensing Ion Channels (ASICs) are a family of proton-gated cation channels that play a role in the sensation of noxious stimuli. Of these, ASIC1a is the only family member that is reported to be permeable to Ca^{2+} , although the absolute magnitude of the Ca^{2+} current is unclear. Here, we used patch-clamp photometry to determine the contribution of Ca^{2+} to total current through native and recombinant ASIC1a receptors. We found that acidification of the extracellular medium evoked amiloride and psalmotoxin 1-sensitive currents in isolated chick dorsal root ganglion neurons and human embryonic kidney cells, but did not alter fura-2 fluorescence when the bath concentration of Ca^{2+} was close to that found in normal physiological conditions. Further, activation of recombinant ASIC1a receptors also failed to produce measurable changes in fluorescence despite of the fact that the total cation current through the over-expressed receptor was ten-fold larger than that of the native channels. Finally, we imaged a field of intact DRG neurons loaded with the Ca^{2+} -sensing dye Fluo-4, and found that acidification increased $[\text{Ca}^{2+}]_i$ in a small population of cells. Thus, although our whole-field imaging data agree with previous studies that activation of ASIC1a receptors can potentially cause elevations in intracellular free Ca^{2+} , our single cell data strongly challenges the view that Ca^{2+} entry through the ASIC1a receptor itself contributes to this response.

1. Introduction

Acid Sensing Ion Channels (ASICs) are a family of epithelial Na^+ Channel/degnerin receptors that conduct cations across the membrane when activated by extracellular hydrogen ions. Upon activation, the channel opens, and sodium moves down its concentration gradient into the cell, resulting in depolarization and an increase in cell excitability. Although most ASICs are impermeable to Ca^{2+} , a number of studies using Ca^{2+} -sensitive indicators suggest that cells expressing the ASIC1a subunit exhibit robust, amiloride-sensitive elevations in $[\text{Ca}^{2+}]_i$ when exposed to extracellular acidification [1–3]. These elevations persist in the presence of antagonists and blockers of downstream Ca^{2+} sources such as voltage-operated Ca^{2+} channels, which implies that Ca^{2+} entry through the ASIC1a is itself responsible for the rise in $[\text{Ca}^{2+}]_i$ [2]. However, estimations of relative Ca^{2+} to Na^+ permeability ($P_{\text{Ca}}/P_{\text{Na}}$) made on the basis of reversal potential experiments range from 0.02 to 0.4 [4–8], and it is difficult to understand how these low permeability channels transduce the relatively large elevations in free intracellular calcium $[\text{Ca}^{2+}]_i$ measured using fluorescent dyes. One possibility is that the $P_{\text{Ca}}/P_{\text{Na}}$ of ASIC1a measured using conventional methods underestimates the ability of Ca^{2+} to permeate the channel. To test this hypothesis, we used the more reliable method of patch-clamp photometry to directly measure the contribution of Ca^{2+} to native and recombinant human ASIC1a (hASIC1a) and chick ASIC1 (cASIC1a). This method employs conventional patch-clamp electrophysiology to record the whole cell current, while simultaneously measuring the fluorescence emission of the Ca^{2+} indicator fura-2 with a photo-multiplier tube. We used a high (2 mM) internal fura-2 concentration to out-compete endogenous cytoplasmic buffers;

this ensures that all the Ca^{2+} that enter the cell bind first to fura-2, and that the change in fluorescence measured by the photo-multiplier tube is directly proportional to Ca^{2+} entry. Calibrating the fluorescence signal allows the fraction of the total current attributed to Ca^{2+} entry ($P_f\%$) to be determined empirically, as successfully demonstrated for ionotropic glutamatergic, purinergic, serotonergic, and cholinergic receptors [9–12]. A major advantage of the dye overload method is that Ca^{2+} entry is measured in bath solutions that contain a physiologic concentration of extracellular calcium ($[\text{Ca}^{2+}]_o$). In contrast, the reversal potential-based method has two disadvantages. First, it estimates PCa/PNa in extracellular solutions containing either abnormally high $[\text{Ca}^{2+}]_o$ (10–112mM) or non-physiologic cations such as N-methyl-d-glucamine. Second, determination of PCa/PNa from reversal potentials is dependent on the assumptions made by the Goldman–Hodgkin–Katz model, which does not necessarily describe ion permeation through all ion channels [13].

In this paper, we use the dye overload method to study native and recombinant ASIC1a channels. Our data fail to support the hypothesis that Ca^{2+} makes a significant contribution to cation flux through the channel pore.

2. Methods

2.1. Isolation of chick DRG neurons

Neurons of the dorsal root ganglia were isolated from chick embryos (day 10) as previously described [14]. Briefly, ganglia were isolated in F12-Ham's media and dissociated with trypsin for 30 min at 37 °C. Trypsin digestion was terminated using 100% fetal bovine serum for 5 min, and the neurons washed twice with B27 supplemented Neurobasal media containing 50 ng/ml nerve growth factor. Neurons were mechanically triturated for dispersal, counted, and seeded onto 22 mm poly-l-lysine coated coverslips in 35 mm dishes for Fluo-4 fluorescence imaging studies. Cultures were grown for 24–72 h in a 5% CO_2 humidified incubator at 37 °C. Culture reagents and fluorescent dyes were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) except where noted otherwise.

2.2. Molecular biology and cell culture

Human embryonic kidney cells (HEK-293) and COS-7 cells (both obtained from ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin, and incubated for 24–48 h at 37 °C in a humidified, 5% CO_2 atmosphere. COS-7 cells were transfected with plasmid(s) encoding the receptor(s) of interest using Lipofectamine LTX and Plus reagents. In control experiments, untransfected COS-7 cell showed no response to protonation (Samways, data not shown). Both HEK and COS-7 cell lines were replated at low density onto poly-l-lysine coated glass coverslips (Gold Seal, Becton, Dickinson Co., Portsmouth, NH) 12–24 h prior to experimentation.

2.3. Patch-clamp photometry

The $P_f\%$ s of acid-evoked currents in chick DRG neurons and HEK293 cells were measured using the dye-overload method [15], as described in Egan and Khakh [10]. Briefly, $P_f\%$ was determined by simultaneously measuring total whole cell current and fluorescence in cells loaded with a high concentration (2 mM) of the calcium-sensitive dye, K5fura-2. Cells were voltage-clamped at -80 mV using borosilicate glass electrodes (1–3 M Ω m)(1B150F, World Precision Instruments, Sarasota, FL), an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), and data acquisition hardware (Instrutech ITC-16, Port Washington, NY) and software (AxoGraphX, Melbourne, Australia). Offline analyses were performed using AxoGraphX and IGOR Pro (Wavemetrics, Lake Oswego, OR). Electrodes contained a solution

of the following composition (in mM): 140 CsCl, 10 tetraethylammonium Cl, 10 HEPES, 2K5fura-2, 4.8 CsOH, pH 7.35. Fura-2 fluorescence intensity (excitation 380 nm; emission 510 nm) was measured using a Model 714 Photomultiplier Detection System (Photo Technology International, South Brunswick, NJ). We controlled the day-to-day variation in the sensitivity of the microscope/PMT by normalizing the fura-2 signal to a “bead unit” (BU), where one BU equaled the average fluorescence of seven individual Carboxy Bright Blue 4.6 μm micro-spheres (Polysciences, Warrington, PA) measured on the day of the experiment. We considered a “measurable change in F_{380} ” to be represented by a signal-to-noise ratio ≥ 2 . Responses smaller than this were difficult to isolate from the background noise detected by the PMT, and thus could not be accurately matched to the time course of the integrated current; a good match is vital because it shows that the ΔF_{380} change is due to Ca^{2+} entry alone. The extracellular solution contained (mM): 140 NaCl, 2 CaCl_2 , 1 MgCl_2 , 10 glucose, and 10 HEPES, titrated to pH 7.4 with 4 NaOH. HEPES was replaced by 2-(N-morpholino) ethanesulfonic acid (MES) in experiments involving acidic (pH 6) buffer. Solutions of different pH were applied using a Perfusion Fast-Step System SF-77 (Warner Instruments, Hamden, CT). The Pf% was calculated as follows:

$$\text{Pf}\% = \frac{Q_{\text{Ca}}}{Q_{\text{T}}} * 100$$

where Q_{T} is the total charge and equal to the integral of the leak-subtracted ATP-gated transmembrane current. Q_{Ca} is the part of Q_{T} carried by Ca^{2+} , and is equal to ΔF_{380} divided by the calibration factor F_{max} determined separately as previously described [10]).

2.4. Fluo-4 imaging experiments

Chick DRG neurons or HEK-293 cells plated on 22 mm coverslips were loaded with fluo-4/AM for 30 min at 22°C. Cells were washed with extracellular buffer and incubated at 37°C for a further 30 min to allow de-esterification of the fluo-4 ester. Images were acquired using a CoolSnap EZ camera (Photometrics, Tucson, AZ), with fluo-4 excited by 488 nm wavelength light, and fluorescence intensity recorded through a 525 nm bandpass filter. Imaging data was analysed using $\mu\text{Manager}$ 1.2 β (Professor R. Vale laboratory, University of California, San Francisco, CA). Fluorescence traces represent the fold fluorescence over basal after background subtraction (F/F_0).

3. Results

3.1. Measurement of Pf% for native ASIC1a receptors

We examined two cell types known to express native ASIC1a receptors, chick DRG neurons and HEK-293 fibroblasts [7,16]. Acid stimulation evoked a robust inward current (140.5 ± 29 pA/pF) in 21 of 21 chick DRG neurons, which is consistent with the presence of functional ASIC1a receptors. This current was transient in nature and inhibited by both the broad-spectrum ASIC channel blocker, amiloride (30 μM) and the selective ASIC1a receptor antagonist psalmotoxin 1 (PcTx1; 30 nM; Peptides International, Louisville KY) (Fig. 1A) [25]. Surprisingly, application of PcTx1 alone caused a small inward current (6.5 ± 3.2 pA/pF) in addition to inhibiting the acid-evoked current. This persistent current is discussed in more detail below.

Stimulation with acid also evoked inward currents in 26 of 53 HEK-293 cells (Fig. 1B). Although these currents were smaller than those recorded in DRG neurons (19.4 ± 2.6 pA/pF), they otherwise resembled the neuronal acid-evoked currents in their transient nature and inhibition by amiloride and PcTx1 with the following exception: PcTx1 had no effect on

membrane holding current of the HEK-293 fibroblasts. Next, we simultaneously measured whole cell current and intracellular fura-2 fluorescence using an extracellular solution containing 2 mM Ca^{2+} and an intracellular solution containing 2 mM fura-2. In 18 of 19 chick DRG neurons in which acidification evoked inward currents, no change in fura-2 fluorescence was observed (Fig. 1C).

As detailed above, the native acid-evoked currents of HEK-293 cells were smaller than those of chick neurons, and thus produced less net charge transfer across the membrane. However, in keeping with the work on chick DRG, no measurable deflection in $F380$ was recorded in 16 of 16 acid-sensitive HEK-293 cells (Fig. 1D). To provide a positive control, we also measured the $Pf\%$ of ATP-evoked currents in HEK-293 cells transiently expressing Ca^{2+} -permeable, purinergic P2X3 receptors, which exhibit a similar rapidly desensitizing response profile to the hASIC1a receptor. Consistent with previous studies, HEK-293 cells expressing the P2X3 receptor responded to ATP (10 μM) with a transient inward current and a measurable deflection in $F380$ [10,17]. The total charge transfer of the ATP-gated current in HEK-293–P2X3 cells equaled 71.5 ± 26 pC/pF, which is in line with that of the chick DRG ASIC1a current (79.0 ± 17 pC/pF), and the $Pf\%$ equaled $5 \pm 0.3\%$ ($n = 4$), consistent with previous results [26].

3.2. $Pf\%$ measurements in COS-7 cells transfected with cASIC1a or hASIC1a DNA

There are two possible explanations for our negative fluorescence results: (1) that native ASIC1a are permeable to Ca^{2+} , but are not expressed in sufficient density for our recording equipment to detect a measurable Ca^{2+} entry; and (2) the native ASIC1a receptors of chick DRG neurons and HEK-293 cells might not represent a pure population of homomeric ASIC1a receptors, and the lack of Ca^{2+} permeability is due to the contribution of Ca^{2+} impermeable subunits, such as ASIC1b, to the native channels expressed in these cells [4].

To examine these possibilities, we next measured the $Pf\%$ of acid-gated currents evoked in COS-7 cells transfected with the cDNAs for either cASIC1a or hASIC1a receptors. COS-7 cells over-expressing cASIC1a and hASIC1a receptors exhibited acid-evoked current densities that were significantly larger (827.2 ± 83 pA/pF, $n = 7$; and 581 ± 95 pA/pF, $n = 16$, respectively) than those recorded for the native receptor types. However, despite of the significant amount of charge crossing the membrane, we failed in all attempts to measure a change in $F380$ resulting from the acid-evoked inward currents (Fig. 2A and B).

We next repeated these $Pf\%$ experiments in an elevated $[\text{Ca}^{2+}]_o$ of 10 mM, in order to test whether a higher chemical driving force for Ca^{2+} entry might reveal a small amount of Ca^{2+} entry. COS-7 cells were co-transfected with plasmids encoding either cASIC1a and P2X3 receptors, or hASIC1a and P2X3 receptors. When stimulated with acid, cells expressing cASIC1a and hASIC1a receptors showed large inward currents (360 ± 52 pA/pF, $n = 3$; and 295 ± 68 pA/pF, $n = 4$; respectively) but, again, no change in fluorescence (Fig. 2C). In contrast, subsequent activation of P2X3 receptors with ATP (10 μM) evoked inward currents and a change in $F380$, which produced a mean $Pf\%$ value of $11.7 \pm 1.9\%$ ($n = 4$) (Fig. 2D). The higher $Pf\%$ of P2X3 receptors in 10 mM $[\text{Ca}^{2+}]_o$ is consistent with the prediction that a rise in $[\text{Ca}^{2+}]_o$ increases $Pf\%$ by making more Ca^{2+} available for transport, and demonstrates the feasibility of this experiment (Fig. 3).

3.3. Even sustained activation of cASIC1a receptors was insufficient to conduct measurable Ca^{2+} entry

We were surprised to find that application of PcTx1 caused a small inward current in chick DRG (see Fig. 1A). This current could be caused by activation of either endogenous ASIC receptors or some other channel. To test the hypothesis that the current flowed through ASIC

channels, we applied PcTx1 (30 nM) to both mock transfected COS-7 cells, and to COS-7 transiently expressing recombinant ASIC1a receptors. Only in cells expressing cASIC1a receptors did application of PcTx1 evoke large, sustained inward current (319 ± 53 pA/pF, $n = 7$) that only partially reversed upon washout. Application of a voltage ramp during the PcTx1-evoked current (from -20 mV to $+80$ mV, 500 ms) revealed a reversal potential of $+69.3$ mV ($n = 3$), which was not significantly different from that of the acid-evoked current ($+66.8$ mV; $n = 4$). This PcTx1-evoked current was not observed in cells transfected with hASIC1a, suggesting that PcTx1 opens ASIC1a channels in a species type-specific manner.

PcTx1 is known to act as an agonist at ASIC1b receptors under mildly acidic conditions, [17], but our data show for the first time that PcTx1 can cause a sustained activation of chick ASIC1a receptors at physiological pH. The persistent nature of the PcTx1-evoked current was surprising, and it gave us the opportunity to see if, by generating an even greater degree of charge transfer over a prolonged period of activation, we might start to see signs of Ca^{2+} entry. However, in three experiments conducted on COS-7 cells expressing cASIC1a receptors, we failed to see fluorescence changes evoked by 5 s of PcTx1 (30 nM), despite the fact that the toxin produced significant charge transfer across the membrane (1439 ± 302 pC/pF, $n = 3$). Again, these experiments suggest that ASIC1a channels are not a significant conduit for Ca^{2+} flux across the membrane.

3.4. Acidification elevates $[\text{Ca}^{2+}]_i$ in a subpopulation of chick DRG neurons

Previous studies report observing acid-evoked elevations in $[\text{Ca}^{2+}]_i$, as assessed from ratiometric fura-2 recordings, in intact mouse cortical and hippocampal neurons, and in rat oligodendrocytes [1–3]. Our data suggest that the cASIC1 receptor of DRG neurons does not transduce a measurable Ca^{2+} current, and thus is unlikely to directly elevate $[\text{Ca}^{2+}]_i$ in intact cells. We next investigated whether exposure to acid affected $[\text{Ca}^{2+}]_i$ in a field of DRG neurons measured using a CCD camera and the Ca^{2+} -sensitive indicator fluo-4-AM. For experiments involving DRG neurons, we added 30 μM ruthenium red to the extracellular buffer to inhibit native TRPV1 receptors [18]. Across seven experiments, 67 of 103 cells responded to a depolarizing pulse of 50 mM KCl with an increase in fluo-4 fluorescence intensity, providing an approximate indication of neuronal versus non-neuronal cells in the DRG culture. In contrast, 33 of 103 cells responded to acid exposure (pH 6, 30 s) with a transient increase in fluorescence (Fig. 4A). Of these, 29 cells responded to both acid stimulation and 50 mM KCl, indicating that approximately 60% of excitable cells in the DRG culture respond to acid exposure. In contrast, in separate experiments conducted in the presence of 100 μM amiloride, only 1 of 58 cells (four separate experiments) responded to acid stimulation (data not shown). 33 of these 58 cells nevertheless responded to 50 mM KCl with an increase in fluo-4 fluorescence. In contrast to the chick DRG neurons, we saw no change in fluo-4 fluorescence in 42 HEK-293 cells recorded in three experiments (Fig. 4B). All 42 cells subsequently responded to ATP, which stimulates Ca^{2+} release and Ca^{2+} entry through an inositol 1,4,5-trisphosphate-dependent pathway, confirming cell viability (data not shown). Finally, we found that HEK-293 cells transfected with TRPV1 cDNA responded to exposure to pH 6 solution with a sustained elevation in $[\text{Ca}^{2+}]_i$, consistent with the high P_f of TRPV1 receptors [19].

4. Discussion

The ASIC1a receptor is reported to exhibit the highest relative Ca^{2+} permeability amongst all the receptors in the ASIC family, but whether direct Ca^{2+} entry through these channels makes a substantial and direct contribution to $[\text{Ca}^{2+}]_i$ homeostasis is debatable. In this paper, we show compelling evidence that neither native nor recombinant ASIC1a receptors conduct measurable Ca^{2+} entry when $[\text{Na}^+]_o$ and $[\text{Ca}^{2+}]_o$ are kept within the physiological range. Our

data strongly imply that direct Ca^{2+} entry through the pore of ASIC1a receptor is not the source of the marked elevations of $[\text{Ca}^{2+}]_i$ observed during acid stimulation in neurons and glia bathed in a saline solution containing physiological concentrations of Ca^{2+} .

Three pieces of evidence have been put forward by previous studies to argue that ASIC1a receptors are Ca^{2+} permeable. First, cells expressing ASIC1a receptors exhibit an inward current upon stimulation with acid when Ca^{2+} is the only extracellular permeable charge carrier [2,3,5]. Second, acid stimulation of ASIC1a receptors evoked robust elevations in $[\text{Ca}^{2+}]_i$ as measured using Ca^{2+} -sensitive dyes in intact neurons and glia [1–3], and in COS-7 cells transfected with hASIC1a cDNA [3]. Third, reversal potential measurements estimate the PCa/PNa to be between 0.02 and 0.4 [4–6,8], which is higher than values obtained for other members of the ASIC receptor family.

The legitimacy of the first piece of evidence is questionable because it is not uncommon for an ion channel that shows very high selectivity for a particular cation to nevertheless freely conduct other cations if the preferred species is absent. For example, K^+ channels conduct Na^+ very well in the absence of K^+ , as do voltage-operated Ca^{2+} channels when Ca^{2+} is not present [13]. Therefore, the finding that Ca^{2+} permeates the ASIC1 receptor in the absence of Na^+ should not be taken as *ipso facto* proof that the channel conducts Ca^{2+} under physiological conditions, because Na^+ could conceivably block Ca^{2+} currents through these receptors in the same way K^+ blocks other cations from passing through K^+ channels.

Regarding the second piece of evidence, we too observed a marked elevation in $[\text{Ca}^{2+}]_i$ upon stimulating a subpopulation of chick DRG neurons with acid, and thus our data concur to a degree with previous studies in native neurons. Nevertheless, two pieces of evidence refute the hypothesis that the elevation in $[\text{Ca}^{2+}]_i$ observed in our imaging experiments was due to Ca^{2+} entry through the ASIC1a receptor itself. First, our patch-clamp photometry experiments show clearly that acid evokes robust inward currents through native and recombinant ASIC1a, but does not cause parallel changes in $F380$, even during sustained activation of cASIC1a or in the presence of elevated $[\text{Ca}^{2+}]_o$. Thus, direct measurement failed to reveal any Ca^{2+} entry through these channels, even though we used a Ca^{2+} indicator that had previously been used to reveal ASIC1a-mediated elevations in $[\text{Ca}^{2+}]_i$ in intact cells [1,2]. Second, only a subpopulation of intact chick DRG neurons loaded with Fluo-4 responded to acid with an elevation in $[\text{Ca}^{2+}]_i$, whereas all neurons responded to acid with an inward current in patch-clamp experiments. Additionally, we did not see any fluo-4-loaded HEK-293 cells respond to acid stimulation with an elevation of $[\text{Ca}^{2+}]_i$, even though 50% of HEK-293 cells responded to acid with inward currents in patch-clamp experiments. Therefore, the occurrence of the acid-evoked elevation of $[\text{Ca}^{2+}]_i$ does not correlate well with the occurrence of the acid-evoked inward current.

With regard to the third piece of evidence, PCa/PNa values determined from reversal potentials measurements of ASIC1a currents are low by comparison to other types of ligand-gated ion channels, leading some investigators to conclude that ASIC1a channels probably don't conduct much Ca^{2+} [4,20]. In *Xenopus laevis* oocytes injected with RNA for rat ASIC1a, the acid-evoked current is reported to have PCa/PNa values of ≈ 0.05 and ≈ 0.4 [4,21]. From data obtained on the acid-evoked current through native ASIC1a expressed in rat neurons, the PCa/PNa of rat ASIC1a receptor is 0.31 and 0.4 [5,8]. The PCa/PNa of the acid-evoked current in HEK-293 cells is reported to be ≈ 0.12 [6] and as low as 0.02 [7]. Our inability to measure Ca^{2+} influx through ASIC1a even in the presence of substantial net charge influx tends to agree with previous data suggesting that the PCa/PNa of these channels is very low.

If ASIC1a receptors conduct very limited Ca^{2+} entry, then how is it that acid stimulation evokes a marked elevation of $[\text{Ca}^{2+}]_i$ in some intact cell types (this paper and [1–3])? Perhaps the most

plausible hypothesis is that activation of ASIC1a receptors does stimulate an elevation of $[Ca^{2+}]_i$ in some cells, but that this occurs through an indirect mechanism involving secondary Ca^{2+} signaling mechanisms. Two earlier studies ruled out a role for voltage-operated Ca^{2+} channels in mediating the Ca^{2+} entry caused by ASIC1a activation. Xiong and co-workers [2] showed that chemical inhibition of voltage-operated Ca^{2+} channels did not attenuate the ASIC1-mediated elevation of $[Ca^{2+}]_i$ in isolated mouse cortical neurons, implying that voltage-operated Ca^{2+} channels were not mediating acid-evoked Ca^{2+} entry. On the other hand, Herrera and co-workers [22] showed that the ASIC1a-mediated elevation of $[Ca^{2+}]_i$ in primary rat cortical neurons was dependent on membrane depolarization, supporting the role of voltage-gated channels in the stimulation of Ca^{2+} entry by ASIC1a receptors.

The data of Yermolaieva and co-workers [3] are particularly interesting because they also studied hASIC1a receptors expressed in COS-7 cells, but reported that stimulation of this receptor elevated $[Ca^{2+}]_i$ in intact cells loaded with fura-2. They report that the elevation of $[Ca^{2+}]_i$ was not due to the release from stores or from the activation of a voltage-dependent source of Ca^{2+} entry, and thus concluded that direct Ca^{2+} entry through hASIC1a receptor itself was the likely cause. Our data provide convincing evidence that the hASIC1a receptor is more-or-less impermeable to Ca^{2+} under physiological conditions, but still leaves two questions that will require further consideration and study. First, if the ASIC1a receptor is, in physiological terms, no more permeable to Ca^{2+} than other ASICs channels such as ASIC1b, then why is it that only ASIC1a has been reported to elevate $[Ca^{2+}]_i$? Second, what voltage-independent Ca^{2+} signaling pathways are being exploited by ASIC1a receptors to give rise to elevations in $[Ca^{2+}]_i$? Whether ASIC1a receptors can directly influence the activity of other Ca^{2+} permeable ion channels is a promising avenue of investigation, particularly given the diversity of such channels expressed in native cells.

An unexpected observation reported here is the finding that the ASIC1a selective inhibitory toxin, PcTx1, has agonist activity at cASIC1a. That PcTx1 behaves as an agonist under certain conditions was first observed by Grunder and co-workers, who reported that the toxin gated ASIC1b receptors at mildly acidic pH ranges [17]. In this paper, we show for the first time that PcTx1 can evoke sustained inward currents through cASIC1a at physiological pH, and that these are only partially reversible upon the removal of PcTx1 within the time course of our experiments. This would seem to support the hypothesis of Grunder and co-workers that PcTx1 interacts with ASIC1 receptors in a state-dependent manner [17], and that the toxin shows a binding preference for the open states of ASIC1b and cASIC1a.

In conclusion, the data presented in this paper demonstrate that Ca^{2+} entry through the ASIC1a channel pore is too negligible to be detected with conventional Ca^{2+} indicators and sensitive light detection apparatus. We cannot definitively rule out the possibility that these channels might conduct physiologically relevant Ca^{2+} entry under conditions not replicated by our experimental methods. For one, we know little about the cation permeability properties of heteromeric ASICs, or whether interaction of ASIC1a with other modulatory proteins might alter permeability. Also, recent work demonstrating that Ca^{2+} selectivity is not necessarily a fixed property of ligand-gated ion channels has urged caution in drawing firm conclusions about ion permeability on the basis of experiments conducted under a limited range of conditions [19,23,24]. Nevertheless, our data strongly advocate for a new perspective on the contribution of ASIC1a to Ca^{2+} homeostasis, and suggest that this channel may largely affect changes in $[Ca^{2+}]_i$ through indirect, but potentially novel, mechanisms.

Acknowledgments

The authors thank Zhiyuan Li for daily discussions, Nico Sturman for advice about $\square$ Manager, and Fred Sherberger and Rick West for discussing bird-eating tarantulas. Meredith Hoge and Kelsey Eckelkamp helped with tissue culture. The plasmids containing the genes for chick ASIC1 and human ASIC1a were gifts kindly provided by Professors Eric

Gouaux (Vollum Institute, OR) and Michael J. Welsh (University of Iowa, IA). We are supported by grants for the NIH.

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Fig. 1. Negligible Ca^{2+} entry through native ASIC1a. (A and B) stimulation of chick DRG neurons or HEK-293 cells with acid (pH 6) evoked inward currents inhibited by amiloride (30 μM) and PcTx1 (30 nM). (C–E) Representative traces showing whole cell current (top), fura-2 fluorescence (gray, bottom), and the integrated current (dashed, bottom). Stimulation of chick DRGs (C) or HEK-293 cells (D) with acid evoked an inward current, but no measurable change in fura-2 fluorescence. (E) HEK-293-P2X3 cells responded to ATP (10 μM) with a transient inward current and a deflection in F_{380} in that followed the time course of the integrated current.

Fig. 2.

Recombinant ASIC1a also failed to mediate measurable Ca^{2+} entry when over-expressed in COS-7 cells. (A and B) Representative traces showing whole cell current (top), fura-2 fluorescence (gray, bottom), and the integrated current (dashed, bottom). Stimulation with acid (pH 6) evoked inward currents in cells expressing chick cASIC1a (A) and hASIC1a (B), but caused no parallel change in F_{380} . (C and D) In COS-7 cells overexpressing P2X3 receptors plus either cASIC1a or hASIC1a elevating $[\text{Ca}^{2+}]_o$ to 10mM still failed to uncover a measurable deflection in F_{380} . In contrast, subsequent stimulation with ATP (10 μM) evoked an inward current paralleled by a deflection in ΔF_{380} (representative trace from a COS-7-cASIC1a/P2X3 cell shown).

Fig. 3.

PcTx1 activates cASIC1a in DRGs and COS-7-cASIC1a cells. A) We observed that PcTx1 (30 nM) evoked a small sustained current when applied to chick DRG neurons. (B and C) Representative traces showing whole cell current (top), fura2 fluorescence (gray, bottom), and the integrated current (dashed, bottom). PcTx1 evoked much larger inward currents in COS-7-cASIC1a that only partially reversed upon washout of PcTx1 (B). However, sustained activation of cASIC1a for 5 s with PcTx1 did not cause a measurable Ca^{2+} entry.

Fig. 4. Acid stimulation evoked an elevation of $[Ca^{2+}]_i$ in some chick DRG neurons. (A and B) Representative traces showing Fluo-4 fluorescence recorded from single cells (gray traces). Black trace shows the average signal. (A) Chick DRG neurons were exposed to acid (pH 6) for 30 s followed by KCl (50 mM) for 30 s. (B) Acid stimulation did not evoke an elevation of $[Ca^{2+}]_i$ in Fluo-4-loaded HEK-293 cells (left), but caused a marked elevation in Ca^{2+} in HEK-293 cells transfected with TRPV1 cDNA (right).