Cell Calcium 45 (2009) 319–325

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

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

Damien S.K. Samways*, Amy B. Harkins, Terrance M. Egan

Department of Pharmacological and Physiological Science, and The Center for Excellence in Neuroscience, Saint Louis University School of Medicine, 1402 South Grand Blvd, St. Louis 63104, MO , USA

ARTICLE INFO

Article history:
Received 21 August 2008
Received in revised form
21 November 2008
Accepted 4 December 2008
Available online 29 January 2009

Keywords:
Ion channel
ASIC1a
Calcium
Permeability
Psalmotoxin 1

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 $[Ca^{2+}]_i$ 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/degenerin 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ [2]. However, estimations of relative Ca^{2+} to Na^+ permeability (P_{Ca}/P_{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 $[Ca^{2+}]_i$ measured using fluorescent dyes. One possibility is that the P_{Ca}/P_{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 (*Pf*%) 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 ($[Ca^{2+}]_o$). In contrast, the reversal potential-based method has two disadvantages. First, it estimates P_{Ca}/P_{Na} in extracellular solutions containing either abnormally high $[Ca^{2+}]_o$ (10–112 mM) or non-physiologic cations such as N-methyl-D-glucamine. Second, determination of P_{Ca}/P_{Na} from reversal potentials is dependent on the assumptions made by the Goldman–Hodgkin–Katz model, which does

* Corresponding author at: Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis 63104, MO, USA. Tel.: +1 314 977 6429; fax: +1 314 977 6411.
E-mail address: samwaysds@slu.edu (D.S.K. Samways).

0143-4160/$ – see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.ceca.2008.12.002

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 μg/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 *Pf*%s of acid-evoked currents in chick DRG neurons and HEK-293 cells were measured using the dye-overload method [15], as described in Egan and Khakh [10]. Briefly, *Pf*% 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, K_5fura-2. Cells were voltage-clamped at −80 mV using borosilicate glass electrodes (1–3 MΩ)(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, 2 K_5fura-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 microspheres (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:

$$Pf\% = \frac{Q_{Ca}}{Q_T} \times 100$$

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 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 were analysed using μManager 1.2β (Professor R. Vale laboratory, University of California, San Francisco, CA). Fluorescence traces represent the fold fluorescence over baseline 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 deflec-