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ASIC1a channels are activated by endogenous protons during ischemia and contribute to synergistic potentiation of intracellular Ca^{2+} overload during ischemia and acidosis

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ABSTRACT

Acidosis accompanying cerebral ischemia activates acid-sensing ion channels (ASIC) causing increases in intracellular calcium concentration ($[Ca^{2+}]_i$) and enhanced neuronal death. Experiments were undertaken in rat cortical neurons to explore the effects of ASIC1a activation on ischemia-induced $[Ca^{2+}]_i$ elevations and whole-cell currents. There was a significant contribution of ASIC1a channels to ischemia-evoked $[Ca^{2+}]_i$ increases at pH 7.4, suggesting that ASIC1a channels are activated by endogenous protons during ischemia. The combination of ischemia and acidosis resulted in synergistic increases in $[Ca^{2+}]_i$ and plasma membrane currents relative to acidosis or ischemia alone. ASIC1a inhibitors significantly blunted $[Ca^{2+}]_i$ increases and a transient current activated by ischemia + acidosis, demonstrating that homomeric ASIC1a channels are involved. However, ASIC1a inhibitors failed to diminish a sustained current activated in response to combined ischemia and acidosis, indicating that acidosis can potentiate ischemia effects through mechanisms other than ASIC1a. The $[Ca^{2+}]_i$ overload produced by acidosis and ischemia was not blocked by tetrodotoxin, 2-amino-5-phosphonopentanoic acid or nifedipine. Thus, acidosis and activation of ASIC1a channels during ischemia can promote $[Ca^{2+}]_i$ overload in the absence of neurotransmission, independent of NMDA receptor or L-type voltage-gated Ca^{2+} channel activation. Postsynaptic ASIC1a channels play a critical role in ischemia-induced $[Ca^{2+}]_i$ dysregulation and membrane dysfunction.

1. Introduction

Glucose and oxygen deprivation associated with brain ischemia initiates a switch in metabolism from aerobic to anaerobic glycolysis to produce cellular energy. The accumulation of lactic acid, the end product of anaerobic glycolysis, leads to acidosis in the ischemic area, and has been detected in both core and penumbral stroke regions in animal models [1]. Recently, these elevations in lactate have been detected in human brains as late as 5 days post-stroke [2]. It has been proposed that this drop in pH results in acidotoxicity that contributes to neuronal injury following cerebral ischemia [3].

One key molecule linking acidosis to neuronal injury appears to be the acid-sensing ion channel 1a (ASIC1a) [4]. ASIC1a are abundant in the central nervous system (CNS) and have a pH of half-maximal activation of ~6.0–6.5 [4,5], which is similar to the pH seen during an ischemic insult [6,7]. Several studies have shown that ASIC1a is activated following cerebral ischemia, and the opening of this channel is linked to neuronal cell death [4,8,9]. Compared to wild-type mice, transgenic mice deficient in ASIC1a have reduced infarct size in response to middle cerebral artery occlusion (MCAO) [4]. Furthermore, pharmacological inhibition of ASIC1a with either the non-selective Na^+ channel blocker, amiloride, or the homomultimeric ASIC1a selective inhibitor psalmotoxin1 (PcTx), diminishes ischemic brain injury [4]. Thus, there is a direct correlation between infarct size, brain acidosis and ASIC activation, suggesting that the acidotoxicity occurring during stroke is in part mediated by ASIC1a channels [4].

The homomeric ASIC1a channel is the only ASIC subtype that is highly permeable to both Na^+ and Ca^{2+} ions [10], and activation of this channel has been suggested to contribute to intracellular calcium overload during ischemia [4]. Previous reports have indicated that the glucose–oxygen deprivation model of ischemia potentiates ASIC1a-mediated currents in neurons [4]. Moreover, it has also been shown that ischemia enhances ASIC1a currents through phosphorylation of the channel by CaMKII, which is activated in response to Ca^{2+} influx through NMDA receptors [8]. Thus, ischemia-induced potentiation of ASIC1a-mediated currents is likely to exacerbate calcium overload during ischemia. Our laboratory has shown that activation of ASIC1a triggers the opening of several downstream channels, including NMDA and AMPA receptors and voltage-gated Ca^{2+} channels, and that these channels are the major contributors

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to the elevations in $[Ca^{2+}]_i$ observed during acidosis [11]. All of these channels are also known to be involved in the disruption of neuronal intracellular $[Ca^{2+}]_i$ homeostasis during ischemia and are likely to be activated by mechanisms independent of ASIC1a. However, it remains to be determined how the convergence of acidosis and ischemia affect the function of these channels, and ultimately $[Ca^{2+}]_i$ homeostasis, during conditions similar to those which occur in vivo during cerebral ischemia.

Experiments were undertaken to ascertain whether acidosis and ischemia interact to produce enhanced elevations in $[Ca^{2+}]_i$ and increased membrane current activation. Ratiometric Ca^{2+} fluorometry experiments demonstrated that acidification of the extracellular solution from pH 7.4 to 6.0 produced a synergistic potentiation in the ischemia-induced rise in $[Ca^{2+}]_i$. Inhibition of ASIC1a channels with either amiloride or PcTx, or increasing the pH buffering capacity of the extracellular solution, significantly decreased elevations in $[Ca^{2+}]_i$ elicited by ischemia alone, suggesting that homomeric ASIC1a channels are activated by endogenous protons during ischemia. Inhibition of these channels also prevented the synergistic potentiation of ischemia-induced $[Ca^{2+}]_i$ overload at pH > 6.5, demonstrating that these channels contribute to the effect of pH. Whereas inhibition of synaptic transmission with tetrodotoxin or block of NMDA receptors and voltage-gated Ca^{2+} channels with 2-amino-5-phosphonopentanoic acid (AP5) and nifedipine, respectively, prevented ischemia-evoked increases in $[Ca^{2+}]_i$, acidification of the extracellular solution (pH 6.0) during acidosis overcame the inhibitory effects of these compounds on $[Ca^{2+}]_i$ overload. Amiloride and PcTx also significantly decreased the initial peak current observed in response to ischemia at pH 6.0, but neither ASIC inhibitor blocked the sustained component of the ischemia + acidosis-induced current.

2. Materials and methods

2.1. Primary rat cortical neuron preparation

Primary cortical neurons from embryonic (E18) rats were cultured as previously described by our laboratory [12]. All procedures were done in accordance with the regulations of the University of South Florida Institutional Animal Care and Use Committee. Cells were used after 10–21 days in culture.

2.2. Calcium imaging measurements

The effects of acidosis and chemical ischemia on intracellular Ca^{2+} concentrations were examined in isolated cortical neurons using fluorescent imaging techniques. Cytosolic free-Ca^{2+} was measured using the Ca^{2+} sensitive dye, fura-2. The membrane permeable ester form of fura-2, fura-2 acetoxymethyl ester (fura-2 AM), was used as we have previously described [13]. Cells plated on coverslips were incubated for 1 h at room temperature in Neurobasal (Invitrogen) medium supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine, or in physiological saline solution (PSS) consisting of (in mM): 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 glucose, and 25 HEPES (pH to 7.4 with NaOH). Both solutions contained 3 μM fura-2 AM and 0.3% dimethyl sulfoxide. The coverslips were washed in PSS (fura-2 AM free) prior to experiments being performed.

2.3. Electrophysiological measurements

Acidosis and ischemia-activated membrane currents were recorded using the protocol previously described by our laboratory [14]. Briefly, neurons plated on glass coverslips were transferred to a recording chamber and membrane currents were amplified, filtered at 240 Hz, digitized at 50 Hz, and acquired using Clampex 9 (Axon), a MultiClamp 700A amplifier and a Digidata 1322A. Electrical access was achieved using the amphotericin B perforated patch method to preserve intracellular integrity of neurons [15]. An amphotericin B stock solution (60 mg/ml in DMSO) was made fresh daily and diluted to 240 μg/ml (0.4% DMSO) in control pipette solution immediately prior to seal formation. The control pipette solution consisted of (in mM): 75 K_2SO_4, 55 KCl, 5 $MgSO_4$, and 10 HEPES (titrated to pH 7.2 with N-methyl-D-glucamine). Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments Inc., Sarasota, FL) using a Sutter Instruments P-87 pipette puller (Novato, CA) and had resistances of 1.0–1.5 MΩ. Access resistance (R_s) was monitored throughout experiments for stable values ≤20 MΩ and was always compensated at 40% (lag, 10 μs). In all electrical recordings, cells were voltage-clamped at −70 mV.

2.4. Solutions and reagents

The control bath solution for all experiments was PSS. For imaging experiments and some of the electrophysiological measurements, drugs were applied in this solution using a rapid application system identical to that previously described [16]. For the remaining electrophysiological experiments, drugs were applied using a multi-barrel manifold with 250 μM tip, positioned within 100 μm of the soma of the cell being studied. Direct ASIC activation was achieved by applying PSS buffered to pH values of 7.0, 6.5 and 6.0 (+/− drug). Chemical ischemia was induced by applying glucose-free PSS containing 4 mM azide (+/− drug) and titrated to pH 7.4, 7.0, 6.5, and 6.0. Individual cells were exposed to ≤4 ischemic, acidic or acidic + ischemic insults with 10-min washes between episodes to prevent rundown of responses [11,12]. Drugs tested for effects on ischemia and/or acidosis were applied for 5 min prior to the ischemia/acidosis unless otherwise noted. All chemicals used in this investigation were of analytical grade. The following drugs were used: 2-amino-5-phosphonopentanoic acid (Tocris Bioscience, Ellisville, MO); psalmotoxin 1 venom (Spider Pharm, Yarnelle, AZ); psalmotoxin 1 peptide (Peptide International, Louisville, KY); tetrodotoxin (Alomone Labs, Jerusalem, Israel); amiloride (Alexis Biochemicals, Lausen, Switzerland); glutamate, nifedipine, sodium-azide (Sigma–Aldrich, St. Louis, MO); and fura-2 AM (Molecular Probes, Eugene, OR).

2.5. Data analysis

Analysis of measured intracellular Ca^{2+} responses was performed using Clampfit 9 (Axon Instruments). Imaging data files collected with SlideBook 4.02 (Intelligent Imaging Innovations, Inc.) were converted to a text format and imported into Clampfit for subsequent analysis. Statistical analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc.). Statistical differences were determined using paired and unpaired *t*-tests for within group and between group experiments, respectively, and were considered significant if $p < 0.05$. For multiple group comparisons either a 1-way or a 2-way ANOVA, with or without repeat measures, were used, as appropriate. When significant differences were determined with an ANOVA, post hoc analysis was conducted using a Tukey test to determine differences between individual groups.

3. Results

3.1. ASIC1a activation contributes to ischemia-evoked $[Ca^{2+}]_i$ increases in cultured rat cortical neurons

During neurotransmission, protons are released along with glutamate but it remains to be resolved whether these protons can