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Evidence for Role of Acid-Sensing Ion Channels in Nucleus Ambiguus Neurons: Essential Differences in Anesthetized versus Awake Rats

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Abstract

Acid-sensing ion channels (ASIC) are widely expressed in several brain regions including medulla; their role in physiology and pathophysiology is incompletely understood. We examined the effect of acidic pH of 6.2 on the medullary neurons involved in parasympathetic cardiac control. Our results indicate that retrogradely-labeled cardiac vagal neurons of nucleus ambiguus are depolarized by acidic pH. In addition, acidic saline of pH 6.2 increases cytosolic Ca^{2+} concentration by promoting Ca^{2+} influx in nucleus ambiguus neurons. *In vivo* studies indicate that microinjection of acidic artificial cerebrospinal fluid (pH 6.2) into the nucleus ambiguus decreases the heart rate in conscious rats, whereas it has no effect in anesthetized animals. Pretreatment with either amiloride or benzamil, two widely used ASIC blockers, abolishes both the *in vitro* and *in vivo* effects elicited by pH 6.2. Our findings support a critical role for ASIC in modulation of cardiac vagal tone and provide a potential mechanism for acidosis-induced bradycardia, while identifying important differences in the response to acidic pH between anesthetized and conscious rats.

Keywords

acidosis; cardiovascular control; heart rate

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Conflict of interest The authors declare that they have no conflicts of interest.

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Introduction

Acid-sensing ion channels (ASIC) are pH-sensitive cation channels activated by extracellular acidosis. ASIC belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily and are susceptible to antagonism by amiloride (Alvarez de la Rosa et al., 2000; Waldmann et al., 1997b). To date, four ASIC genes (*ASIC1-4*) and six channel subunits have been identified (Wemmie et al., 2013). The subunits may assemble to form both homomeric and heteromeric channels, with different pH-dependency and desensitization kinetics, mediating distinct acid-evoked currents (Hesselager et al., 2004).

ASIC are widely expressed in the central and peripheral nervous system (Price et al., 1996; Waldmann et al., 1997b). Acid-sensing is critical for sensory processing of pain and taste (Linguеglia, 2007); however, the physiological and pathophysiological roles of ASIC in the central nervous system are just beginning to be understood (Chu and Xiong, 2012).

The brain pH is tightly controlled and maintained around 7.3-7.4 (Ritucci et al., 1998). Protons modulate physiological processes such as synaptic transmission, as they are co-released with neurotransmitters in the synaptic cleft (Wang and Xu, 2011). Inflammation, ischemic stroke, traumatic brain injury, and epileptic seizure are conditions in which protons over-accumulate, leading to brain acidosis (Chu and Xiong, 2012). Several pathological conditions associated with acidosis are accompanied by autonomic dysregulation and bradyarrhythmia (Hotta et al., 2009; Mameli et al., 2001; Mameli et al., 2006; Saritemur et al., 2013); nevertheless, the function of ASIC in central cardiovascular control has not been explored.

Cardiorespiratory homeostasis is regulated by central pH sensitivity (Nattie, 2000; Spyer & Gourine, 2009). ASICs in the rat nucleus tractus solitarius may mediate chemosensitive responses involved in central respiratory control (Huda et al., 2012). Genetic deletion of ASIC2 leads to hypertension and increased heart rate in conscious mice, due to an enhanced sympathetic and depressed parasympathetic cardiovascular control (Lu et al., 2009). Since cardiac parasympathetic tone is primarily modulated by a population of neurons from nucleus ambiguus (Mendelowitz, 2004), and human (Price et al., 1996) and rat medulla express ASIC isoforms (Huda et al., 2012), we examined the effect of acidic pH (6.2) on cardiac vagal neurons of ambiguus neurons.

Materials and methods

Ethical approval

Animal protocols were approved by the Institutional Animal Care and Use Committee from Thomas Jefferson University, Temple University and Rutgers University. All efforts were made to minimize the number of animals used and their suffering.

Chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. The aCSF composition (in mM) was: 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, pH 7.4 and pH 6.2.

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Animals

Neonatal and adult rats (Charles River Laboratories, Wilmington, MA) were used in this study. Neonatal (1-2 days old) rats of either sex were used for retrograde tracing and neuronal culture, and adult male rats (250-300 g) were used for cardiovascular measurements.

Neuronal labeling and culture

Preganglionic cardiac vagal neurons of nucleus ambiguus were retrogradely labeled by intrapericardial injection of rhodamine [X-rhodamine-5-(and-6)-isothiocyanate; 5(6)-XRITC], 40 μl, 0.01%, (Invitrogen, Carlsbad, CA), as reported (Brailoiu et al., 2013a; Brailoiu et al., 2013b; Brailoiu et al., 2013c). Medullary neurons were dissociated and cultured 24 h after rhodamine injection, as previously described (Brailoiu et al., 2013a; Brailoiu et al., 2013b; Brailoiu et al., 2013c). In brief, the brains were quickly removed and immersed in ice-cold Hanks' balanced salt solution (HBSS; Mediatech, Manassas, VA, USA). Neonate rats were euthanized by decapitation. The ventral side of the medulla (containing nucleus ambiguus) was dissected, minced, and the cells were subjected to enzymatic and mechanical dissociation. Cells were plated on glass coverslips in Neurobasal-A medium (Invitrogen) containing 1% GlutaMax (Invitrogen), 2% antibiotic-antimycotic (Mediatech), and 10% fetal bovine serum. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cytosine β-arabino furanoside (1 μM) was added to the culture to inhibit glial cell proliferation (Schoniger et al., 2001). Cellular analyses were performed only on rhodamine-labeled neurons.

Calcium imaging

Measurements of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ were performed as previously described (Brailoiu et al., 2013a; Brailoiu et al., 2013b; Brailoiu et al., 2013c). Briefly, cells were incubated with 5 μM Fura-2 AM (Invitrogen) in HBSS at room temperature for 45 min, and washed with dye-free HBSS. Coverslips were mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc., Melville, NY), equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). During the experiments the Perfect Focus System was activated. Fura-2 AM fluorescence (emission 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired/analyzed using NIS-Elements AR 3.1 software (Nikon). After appropriate calibration with ionomycin and $CaCl_2$, and Ca^{2+} free and EGTA, respectively, the ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentrations (Grynkiewicz et al., 1985). Upon transfer to the recording chamber, the solutions being tested were applied after one minute recording of the baseline. The solution was changed with the use of a peristaltic pump (Gilson MiniPulse 3, Middleton, WI) at speed of 2 mL/min.

Measurement of membrane potential

The relative changes of neuronal membrane potential were evaluated using bis-(1,3-dibutylbarbituric acid)-trimethine-oxonol, $DiBAC_4(3)$, a voltage-sensitive dye, as reported

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(Brailoiu et al., 2013b; Brailoiu et al., 2013c). Upon membrane hyperpolarization, the dye concentrates in the cell membrane, leading to a decrease in fluorescence intensity, while depolarization induces the sequestration of the dye into the cytosol, resulting in an increase of the fluorescence intensity. Cultured hypothalamic neurons were incubated for 30 min in HBSS containing 0.5 mM $DiBAC_4(3)$ and the fluorescence monitored at 0.17 Hz, excitation/emission: 480 nm/540 nm. Calibration of $DiBAC_4(3)$ fluorescence following background subtraction was performed using the Na^+-K^+ ionophore gramicidin in Na^+-free physiological solution and various concentrations of K^+ (to alter membrane potential) and N-methylglucamine (to maintain osmolarity) (Brauner et al., 1984). Under these conditions, the membrane potential was approximately equal to the K^+ equilibrium potential determined by the Nernst equation. The intracellular K^+ and Na^+ concentration were assumed to be 130 mM and 10 mM, respectively (Brauner et al., 1984).

Surgical procedures

Adult male Sprague Dawley rats were anesthetized with a mixture of ketamine hydrochloride (100-150 mg/kg) and acepromazine maleate (0.2 mg/kg) as reported (Benamar et al., 2010; Brailoiu et al., 2013b; Brailoiu et al., 2013c). Animals were placed into a stereotaxic instrument; a guide C315G cannula (PlasticsOne, Roanoke, VA) was bilaterally inserted into the nucleus ambiguus. The stereotaxic coordinates for identification of nucleus ambiguus were: 12.24 mm posterior to bregma, 2.1 mm from midline and 8.2 mm ventral to the dura mater. A C315DC cannula dummy (PlasticsOne) was used to prevent contamination. For transmitters implantation, a 2 cm-long incision was made along the linea alba. A calibrated transmitter (E-mitters, series 4000; Mini Mitter, Sunriver, OR) was inserted in the intraperitoneal space, as previously described (Benamar et al., 2010; Brailoiu et al., 2013b; Brailoiu et al., 2013c). Subsequently, the abdominal musculature and dermis were sutured independently, and animals returned to individual cages.

Telemetric heart rate monitoring

The signal generated by transmitters was collected via series 4000 receivers (Mini Mitter, Sunriver, OR), as previously described (Brailoiu et al., 2013b; Brailoiu et al., 2013c). VitalView™ software (Mini Mitter, Sunriver, OR) was used for data acquisition. Each data point represents the average of heart rate per 30 s.

Non-invasive blood pressure measurement

In rats with cannula inserted into the nucleus ambiguus, blood pressure was non-invasively measured using a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT), as described (Brailoiu et al., 2013c). One week after the insertion of the cannula, rats were exposed to handling and training every day for 1 week. The maximum occlusion pressure was 200 mm Hg, minimum pressure 30 mm Hg and deflation time 10 s. Two measurements were done per 30 s (one cycle), and the average was used to calculate heart rate, systolic, diastolic and mean arterial blood pressure. Ten acclimatization cycles were done before starting the experiments.

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Microinjection into nucleus ambiguus in conscious rats

One week after surgery (telemetric studies), or after another week of training (tail-cuff measurements), bilateral microinjections into the nucleus ambiguus were carried out using the C315I internal cannula (33 gauge, PlasticsOne), without animal handling. In the tail-cuff method, trained rats were in the animal holder for the duration of the experiment. For recovery, at least two hours were allowed between two injections. Injection of L-glutamate (5 mM, 50 nL with Neuros Hamilton syringe, Model 7000.5 KH SYR) was used for the functional identification of nucleus ambiguus (Brailoiu et al., 2013a; Brailoiu et al., 2013b).

Microinjection into nucleus ambiguus and cardiovascular rate monitoring in anesthetized animals

Adult male Wistar rats were anaesthetized by inhalation of isoflurane (2–3% in 100% oxygen), the trachea was cannulated, and animals were artificially ventilated, as described (Brailoiu et al., 2013a; Chitravanshi et al., 2012). Urethane (1.2–1.4 g/ kg) was injected intravenously in a cannulated femoral vein. Rectal temperature was maintained at 36.5 ± 0.5°C. Upon cannulation of a femoral artery, pulsatile arterial pressure, mean arterial blood pressure and heart rate were measured using a 1401 A/D converter and Spike2 software (Cambridge Electronic Design, Cambridge, UK). For microinjection in the nucleus ambiguus, the rats were placed in a prone position in a stereotaxic instrument with bite bar 18 mm below the interaural line. Multi-barrel glass micropipettes (tip size 20–40 μm) were mounted on a micromanipulator, and connected to one of the channels on a picospritzer. One barrel was filled with L-glutamate (5 mM), the second barrel was filled with aCSF of pH 7.4, and the third barrel was filled with aCSF of pH 6.2. The following coordinates were used for the identification of the nucleus ambiguus: 0.3 caudal to 1.1 mm rostral and 1.8–2.0 mm lateral to the calamus scriptorius and 2.0–2.4 mm deep from the dorsal medullary surface; stimulation of this site has been shown to elicit the most prominent bradycardic response. The correct placement of the pipette assembly was determined by evaluating the bradycardic response to glutamate (5 mM, 30 nL). The volumes were pressure ejected (30–35 psi) and visually confirmed by the displacement of fluid meniscus in the barrel containing the solution. At the end of the experiments, anesthetized rats were euthanized by CO_2 inhalation.

Statistical analysis

Data were expressed as mean ± standard error of mean. One-way ANOVA followed by *post hoc* analysis using Bonferroni and Tukey tests was used to evaluate significant differences between groups; $P < 0.05$ was considered statistically significant.

Results

Acidic saline depolarizes cardiac preganglionic neurons of nucleus ambiguus

Cultured cardiac vagal neurons of nucleus ambiguus had a mean resting membrane potential of – 55.4 ± 0.04 mV (n = 53). Acidic saline (HBSS, pH 6.2) depolarized cardiac vagal neurons; a representative example is shown in Fig. 1a. The mean amplitude of the depolarization was 4.7 ± 0.36 mV (n = 6; Fig. 1b). Pretreatment of neurons with ASIC

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inhibitors amiloride (100 μM, 20 min) or benzamil (100 μM, 20 min) prevented the depolarizing effect of acidic saline (V was 0.82 ± 0.19, and 0.67 ± 0.16, n = 6, respectively; Fig. 1a, b). In the presence of urethane (1.2 mg/mL, 20 min), acidic saline induced an insignificant effect on membrane potential (V = 0.68 ± 0.17 mV; n = 6; Fig. 1a, b). ASIC inhibitors at pH 7.4 or urethane at pH 7.4 had no effect on the mean resting membrane potential of rhodamine-labeled neurons.

Acidic saline increases intracellular $[Ca^{2+}]_i$ in nucleus ambiguus neurons

Application of acidic saline (pH 6.2) to cardiac vagal neurons triggered a fast and sustained elevation of $[Ca^{2+}]_i$, with a mean amplitude of 281 ± 3.4 nM (n = 6) at the peak of the response (Fig. 2 a, b). Ca^{2+}-free saline (pH 6.2) did not significantly affect cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$ was 2 ± 1.7 nM, n = 6, Fig. 2a, b). In neurons pretreated with ASIC inhibitors, acidic saline (pH 6.2) produced negligible Ca^{2+} responses; in the presence of amiloride (100 μM, 20 min), $[Ca^{2+}]_i$ was 8 ± 2.1 nM, n = 6, Fig. 2a, b, e), and in the presence of benzamil (100 μM, 20 min), $[Ca^{2+}]_i$ was 11 ± 1.9 nM, n = 6, Fig. 2a, b, f). Likewise, urethane (1.2 mg/mL, 20 min) prevented the Ca^{2+} response of cardiac vagal preganglionic neurons to acidic saline ($[Ca^{2+}]_i$ was 9.7 ± 2.3 nM, n = 6, Fig. 2a, b, g). Representative examples of Ca^{2+} responses are shown in Fig 2a, the comparison of the mean amplitude of the response in Fig. 2b and examples of changes in fluorescence 340/380 ratio are shown in Fig. 2c-g. Application of pH 7.4 solutions of either ASIC inhibitors or urethane had no effect on the baseline $[Ca^{2+}]_i$ of these neurons.

Microinjection of acidic aCSF into nucleus ambiguus produces bradycardia in conscious rats

In conscious, freely moving rats, bearing cannula implanted into the nucleus ambiguus, microinjection of control aCSF (pH 7.4, 50 nL) did not significantly affect the heart rate, monitored telemetrically or by the tail-cuff method. The correct placement of the cannula was indicated by the bradycardic response produced by microinjection of L-glutamate (L-Glu, 5 mM, 50 nL), without an effect on blood pressure, as previously reported (Brailoiu et al., 2013a; Brailoiu et al., 2013b; Chitravanshi et al., 2012). Two hours after L-Glu administration, microinjection of acidic aCSF (pH 6.2, 50 nL) produced a rapid decrease in heart rate, which slowly returned to basal levels; this response was not accompanied by a change in blood pressure. . Concomitant microinjection of an ASIC blocker (either amiloride or benzamil; both 100 μM, 50 nL) and aCSF (pH 6.2), prevented the effect of acidic aCSF. Representative examples are shown in Fig. 3a. L-Glu decreased the heart rate by 84 ± 7 beats per minute (bpm) (telemetry) and by 81 ± 3 bpm (tail-cuff), respectively (Fig. 3b) whereas aCSF (pH 6.2) reduced the heart rate by 27 ± 3 bpm (telemetry) and by 28 ± 3 bpm (tail cuff), respectively, indicating a good correlation between the two methods (n = 5 rats/group). Microinjection into the nucleus ambiguus of aCSF (pH 7.4) did not produce a significant change in heart rate as compared to the basal heart rate (4 ± 2 bpm in both paradigms; Fig. 3b). Likewise, in the presence of ASIC blockers, the effect of aCSF (pH 6.2) was negligible, measuring –5 ± 4 bpm in animals pretreated with amiloride and – 6 ± 3 bpm in rats pretreated with benzamil (telemetry, Fig. 3b) (n = 5).

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Lack of effects of acidic aCSF on heart rate in anesthetized animals

In animals anesthetized with isoflurane and urethane, L-glutamate (L-Glu, 5mM, 30 nL) microinjected into the nucleus ambiguus transiently decreased the heart rate by 67 ± 4 bpm (n = 5 rats) without any effect on blood pressure, indicating the correct placement of cannula (Brailoiu et al., 2013a; Brailoiu et al., 2013b; Brailoiu et al., 2013c; Chitravanshi et al., 2012). Two hours after L-glutamate administration, microinjection of acidic aCSF (pH 6.2; 30 nL) did not significantly decrease the heart rate, the overall effect measuring − 3.2 ± 0.9 bpm (n = 5), similar to that of control aCSF (pH 7.4; 30 nL), which measured − 2.8 ± 0.7 bpm (n = 5) (Fig. 4 a-c).

Discussion

Increasing evidence supports ASIC involvement in both physiological and pathological neuronal processes, including synaptic transmission, synaptic plasticity, learning, memory, nociception, depression, and acidosis-related neurotoxicity (Wemmie et al., 2002; Wemmie et al., 2013). During synaptic transmission, protons are co-released with neurotransmitters, leading to extracellular acidification of synaptic cleft with modulatory role on synaptic activity (Miesenbock et al., 1998; Wang and Xu, 2011).

Brain acidosis may have a respiratory or metabolic cause; in addition, it may be produced by increased release of acidic vesicles, during seizures (Rehncrona, 1985). Autonomic dysregulation and bradyarrythmias occur in many pathological conditions including ischemic stroke, inflammation, seizure and traumatic injury (Hotta et al., 2009; Mameli et al., 2001; Saritemur et al., 2013). Seizures may elicit brain acidosis and vagally-mediated bradyarrhythmias that may lead to sudden death (Hotta et al., 2009). Moreover, bradycardia and metabolic acidosis are concurrent in propofol-infusion syndrome (Fudickar and Bein, 2009). Since the brain pH may reach values between 6.0 and 6.8 locally, at the synaptic level, or in ischemia, seizure and Alzheimer’s disease (Wang and Xu, 2011), we tested the effect of acidic pH (6.2) on the activity of cardiac vagal neurons of nucleus ambiguus, a key site for heart rate regulation (Mendelowitz, 2004).

Our results indicate that acidic saline (pH 6.2) depolarized cardiac vagal neurons of nucleus ambiguus. Moreover, acidic saline elevated $[Ca^{2+}]_i$ of cardiac vagal neurons of nucleus ambiguus; the Ca^{2+} response was abolished in Ca^{2+}-free saline of pH 6.2. This indicates contingency of the Ca^{2+} response elicited by acidic pH on a Ca^{2+} entry process, likely triggered by membrane depolarization. ASIC1A and ASIC3, subunits activated by pH 6.2, were identified in CNS (Wemmie et al., 2013). Similarly, previous reports indicate that ASIC1A subunit modulates neuronal functions by eliciting Ca^{2+} entry and membrane depolarization (Wemmie et al., 2013). On the other hand, a reduced acid-evoked Ca^{2+} entry was reported in ASIC1A-knockout mice (Wemmie et al., 2013). We note that both the depolarization and the Ca^{2+} response evoked by acidic saline presented a sustained phase, which is consistent with the observation that some ASIC subunits, such as ASIC3 – which is expressed in the brainstem (Wu et al., 2012; Wu et al., 2010) – mediate an inward current that has both a rapidly inactivating and a sustained component (Salinas et al., 2009; Waldmann et al., 1997a); this sustained component is particularly apparent when pH is dropped below 6.5 (Salinas et al., 2009). The amplitude of the depolarization induced at pH

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6.2 in rhodamine-labeled cardiac vagal neurons of nucleus ambiguus is rather small as compared with the ASIC-mediated depolarization in other types of neurons. However, since $DiBAC_4(3)$ is a slow-response voltage-sensitive dye, fast changes is membrane potential and neuronal firing induced by acidic saline may have been overlooked in the present study. Accordingly, a drop in pH from 7.4 to 6.3 elicited single action potential firing in sensory neurons, followed by a sustained plateau of amplitude comparable to that reported here (Deval et al., 2003).

Pretreatment with amiloride, a widely used ASIC blocker (Kellenberger and Schild, 2002; Waldmann et al., 1997b), or with its derivative benzamil, prevented the effect of acidic pH (6.2) on neuronal membrane potential and $[Ca^{2+}]_i$, indicating that these effects are mediated by ASIC. Despite routine use as inhibitors of DEG/ENaC family members (Kellenberger and Schild, 2002), both amiloride and benzamil may have additional off-target effects such as inhibition of the Na^+/H^+ exchanger (Ritucci et al., 1997) or T-type Ca^{2+} channels (Tang et al., 1988). Benzamil has a ten times higher potency than amiloride on DEG/ENaC channels as compared with amiloride (Kleyman and Cragoe, 1988), but it also inhibits the Na^+/H^+ exchanger and may block L-type Ca^{2+} channels (Garcia et al., 1990). However, the Na^+/H^+ exchanger is fully inhibited when extracellular pH drops to acidic levels (Ritucci et al., 1998; Ritucci et al., 1997). In addition, the Ca^{2+} current in cardiac preganglionic neurons of nucleus ambiguus is mediated nearly entirely by P/Q channels (Irnaten et al., 2003), which are not affected by either amiloride or benzamil. Viewed in this context, the effects of amiloride and benzamil presented in this study are most likely attributed to ASIC inhibition.

It is worth noting that both neurons and astrocytes express ASICs. In the *in vitro* studies we tested the effects of acidic pH on isolated neurons; several steps were taken to ensure a neuronal culture (filtration, culture medium which promotes pure populations of neuronal cells and addition of a pharmacological inhibitor of glial proliferation). On the other hand, in the *in vivo* studies, using microinjection in the nucleus ambiguus, we cannot exclude the involvement of astrocytes, in addition to neurons, to the response.

We also noted that urethane suppressed the depolarization and the Ca^{2+} increase induced by acidic saline (pH 6.2) in cardiac vagal neurons of nucleus ambiguus. Similarly, previous reports found that urethane depressed action potential discharge of cortical neurons *in vitro* (Sceniak and Maciver, 2006) or suppressed the spontaneous cortical Ca^{2+} waves *in vivo*, in neonate rats (Adelsberger et al., 2005). In addition, in cortical pyramidal neurons, urethane reduced the Ca^{2+} spike activity of dendrites (Potez and Larkum, 2008).

Microinjection of acidic aCSF (pH 6.2) in nucleus ambiguus in conscious, freely-moving rats, produced bradycardia. The decrease in heart rate was abolished by pretreatment with amiloride or benzamil supporting ASIC activation as an underlying mechanism. On the other hand, in rats anesthetized with isoflurane and urethane, microinjection of acidic aCSF in nucleus ambiguus had no effect on heart rate. This notable difference in the response elicited by acidic aCSF in conscious versus anesthetized rats is in agreement with previous reports indicating that isoflurane suppresses the activity of cardiac vagal neurons of nucleus ambiguus (Toader et al., 2011). Consistently, the *in vitro* effects of urethane on acidic saline-induced depolarization and Ca^{2+} increase were also inhibitory.

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Another important observation is the discrepancy in the time-course of the bradycardic response to glutamate microinjection in the nucleus ambiguus of conscious versus anesthetized animals. In awake rats, the decrease in heart rate produced by glutamate lasted for several minutes, which is in agreement with our previous reports (Brailoiu et al., 2013b; Brailoiu et al., 2014; Brailoiu et al., 2013c); in anesthetized rats, the glutamate-induced bradycardia was short-lived and receded within seconds, a finding likewise consistent with other studies (Brailoiu et al., 2013a; Chitravanshi et al., 2012; Marchenko and Sapru, 2003). This difference in the kinetics of glutamate-mediated responses in the nucleus ambiguus may also be attributable to anesthesia.

General anesthetics such as isoflurane and propofol, at therapeutic concentrations, enhance $GABA_A$ergic inhibitory neurotransmission in cardiac vagal neurons of nucleus ambiguus (Wang, 2009; Wang et al., 2004). Isoflurane reduces the Ca^{2+} current of parasympathetic neurons isolated from bullfrog hearts (Hirota et al., 1999) and has been reported to inhibit both high- and low-voltage activated Ca^{2+} channels in hippocampal pyramidal neurons (Study, 1994).

These results further support the value of cardiovascular assessment in conscious rats, to avoid the complex effects of anesthetics.The differences noted in the response in conscious versus anesthetized rats support also mechanisms that may impact intraoperative cardiovascular regulation in humans, due to the anesthetics.

Previous studies indicate that genetic deletion of ASIC2 leads to hypertension and increased heart rate in conscious mice, via an enhanced sympathetic and depressed parasympathetic cardiovascular control (Lu et al., 2009). However, since ASIC2A subunits have higher sensitivity for more acidic pH (4.5-4.9) (Wemmie et al., 2013) than that tested here (pH 6.2), and ASIC2B subunits do not form pH-sensitive homomeric channels, different subunits may be involved in the response reported here.

Taken together, our results support the ASIC-mediated modulation of cardiac parasympathetic tone via activation of cardiac vagal neurons of nucleus ambiguus. Since ASIC activation occurs in a variety of physiological and pathophysiological conditions, the significance of ASIC-mediated increase in cardiac vagal tone remains to be determined.

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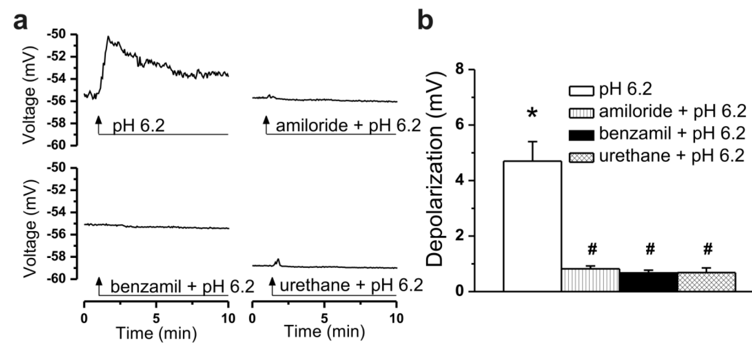

Fig. 1. Acidic saline of pH 6.2 depolarizes cardiac vagal neurons of nucleus ambiguus

a, Representative traces illustrating changes in membrane potential of neurons upon administration of acidic saline (pH 6.2) in the absence and presence of ASIC inhibitors amiloride and benzamil or in the presence of urethane. **b**, Acidic saline produced a mean depolarization of 4.7 ± 0.36 mV in cardiac vagal neurons of nucleus ambiguus; the effect was abolished by pretreatment with amiloride, benzamil or urethane. $P < 0.05$ compared to basal levels (*) or to HBSS pH 6.2 (#)

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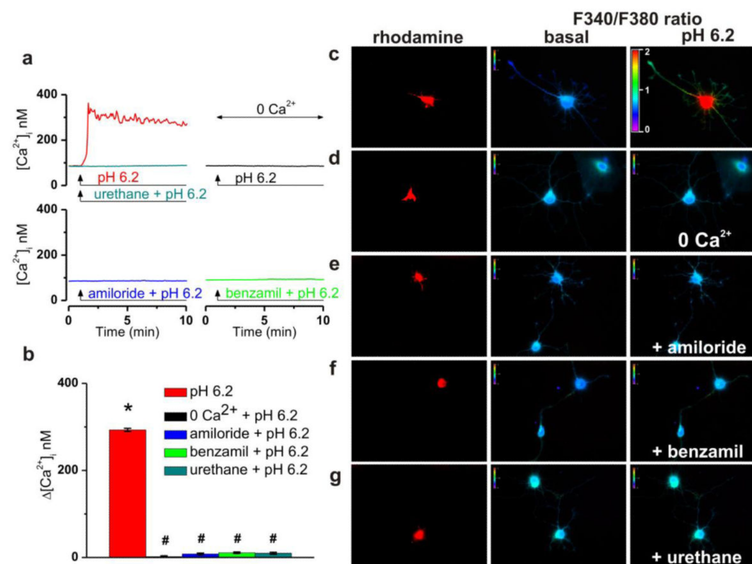

Fig. 2. Acidic saline of pH 6.2 elevates $[Ca^{2+}]_i$ of nucleus ambiguus neurons by triggering Ca^{2+} entry

a, Representative recordings of the Ca^{2+} responses of rhodamine-labeled cardiac vagal neurons indicating an increase in $[Ca^{2+}]_i$ elicited by Ca^{2+}-containing saline (pH 6.2, top left panel, red trace), which was abolished in presence of urethane (top left, dark cyan); in absence of extracellular Ca^{2+} (top right); or in the presence of ASIC blockers amiloride (100 μM, bottom left) or benzamil (100 μM, bottom right). **b**, Comparison of the mean amplitude of Ca^{2+} responses triggered by acidic saline (pH 6.2) in the conditions mentioned in **a**; $P < 0.05$ compared to basal levels (*) or to Ca^{2+}-containing saline pH 6.2 (#). **c-g**, Fura-2 AM fluorescence ratio (340/380 nm) of rhodamine-labeled neurons, before and after application of Ca^{2+}-containing saline of pH 6.2 (**c**), Ca^{2+}-free saline pH 6.2 (**d**), or of Ca^{2+}-containing saline (pH 6.2) in presence of amiloride (**e**), benzamil (**f**) or urethane (**g**). The ratio scale is magnified in **c.**

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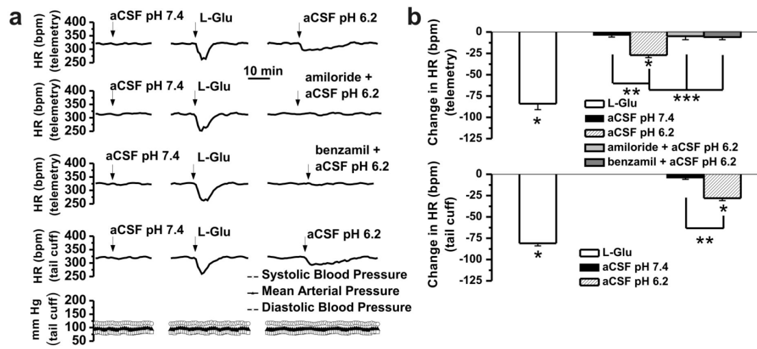

Fig. 3. Microinjection of acidic aCSF (pH 6.2) into the nucleus ambiguus elicits bradycardia in conscious rats

a, Representative examples of telemetric measurements of heart rate after microinjection in nucleus ambiguus of control aCSF (pH 7.4), L-glutamate (L-Glu, 5 mM) and acidic aCSF (pH 6.2) in the absence and presence of ASIC blockers amiloride or benzamil. Similar effects on heart rate were observed using the tail-cuff method; the blood pressure remained unchanged. **b**, Comparison of the effects on heart rate observed in the treatment conditions mentioned in **a**; the decrease in heart rate induced by microinjection of L-Glu or aCSF (pH 6.2) were similar in telemetric and tail-cuff methods; $P < 0.05$ as compared to basal heart rate (*), to control aCSF of pH 7.4 (**) or to acidic aCSF of pH 6.2 (***). Abbreviations: HR, heart rate; bpm, beats per minute

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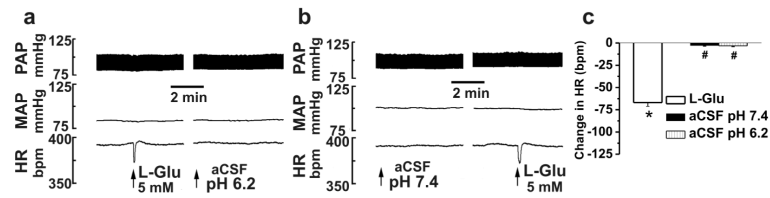

Fig. 4. Microinjection of acidic aCSF (pH 6.2) into the nucleus ambiguus does not affect heart rate in anesthetized rats

a-b, Illustration of representative experiments where microinjection of acidic aCSF (pH 6.2) or of control aCSF (pH 7.4) into the nucleus ambiguus of anesthetized rats did not affect the heart rate. L-glutamate (L-Glu, 5 mM) elicited bradycardia, indicating the correct placement of cannula into the nucleus ambiguus. The pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) were unaffected by either treatment. **c**, Comparison of the effects on heart rate (HR) of microinjection in nucleus ambiguus of L-Glu, aCSF (pH 6.2) and aCSF (pH 7.4) in anesthetized rats. Abbreviations: bpm, beats per minute; $P < 0.05$ as compared to basal heart rate (*) or with the effect of L-Glu (#).