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A HCO_3^--dependent mechanism involving soluble adenylyl cyclase for the activation of Ca^{2+} currents in locus coeruleus neurons ☆

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ABSTRACT

Hypercapnic acidosis activates Ca^{2+} channels and increases intracellular Ca^{2+} levels in neurons of the locus coeruleus, a known chemosensitive region involved in respiratory control. We have also shown that large conductance Ca^{2+}-activated K^+ channels, in conjunction with this pathway, limits the hypercapnic-induced increase in firing rate in locus coeruleus neurons. Here, we present evidence that the Ca^{2+} current is activated by a HCO_3^--sensitive pathway. The increase in HCO_3^- associated with hypercapnia activates HCO_3^--sensitive adenylyl cyclase (soluble adenylyl cyclase). This results in an increase in cyclic adenosine monophosphate levels and activation of Ca^{2+} channels via cyclic adenosine monophosphate-activated protein kinase A. We also show the presence of soluble adenylyl cyclase in the cytoplasm of locus coeruleus neurons, and that the cyclic adenosine monophosphate analogue db-cyclic adenosine monophosphate increases $Ca^{2+}{}_i$. Disrupting this pathway by decreasing HCO_3^- levels during acidification or inhibiting either soluble adenylyl cyclase or protein kinase A, but not transmembrane adenylyl cyclase, can increase the magnitude of the firing rate response to hypercapnia in locus coeruleus neurons from older neonates to the same extent as inhibition of K^+ channels. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

1. Introduction

Neurons that are sensitive to CO_2/H^+ exist in numerous brain regions and contribute to various functions and disorders including the control of breathing, learning and memory, depression and panic disorders [8,30,41,45]. These CO_2/H^+-sensitive neurons are referred to as chemosensitive and the ability of a neuron to respond in this way is generally attributed to the presence of acid-sensitive ion channels on its surface membrane [30,31] We have focused on chemosensitive neurons within one brain stem area, the locus coeruleus (LC). There is considerable evidence that chemosensitive neurons from the LC play an important role in the hypercapnic ventilatory response (recently reviewed in [14]). Early studies showed that focal acidification of the LC alone resulted in increased ventilation [6], showing that the LC could drive increased breathing. Further, lesioning a large percentage of LC neurons resulted in a marked decrease in the hypercapnic ventilatory response to inspired CO_2 [2,23]. Finally, a high percentage of LC neurons have been shown to be chemosensitive [11,12,29] and LC neurons in culture were shown to exhibit intrinsic chemosensitivity [22]. Taken together, these studies point to chemosensitive LC neurons as playing an important role in the control of breathing and in the ventilatory response to inspired hypercapnia.

The chemosensitive response to hypercapnia could be due to the sensing of changes of molecular CO_2, to changes of intracellular and/or extracellular pH, or to changes of HCO_3^- in response to hypercapnia. Recently, evidence has been presented that CO_2 itself could be directly sensed in leptomeninges and glial cells in the ventral medullary surface [19]. The mechanism probably involves CO_2 directly modifying connexin hemichannels by forming a carbamate bridge between two residues that favors the open state in the hemichannel [26]. Numerous other studies have focused on the ability of changes of intracellular or extracellular pH during hypercapnia to alter the activity of ion channels [31]. Chemosensitive LC neurons have been shown to contain a variety of pH-sensitive channels, including inward rectifying K^+ channels [29], transient A currents and delayed rectifying K^+ currents [24], TASK channels [1] and TRP channels [9]. Acidification alters these channels in such a way that LC neurons depolarize and increase their firing rate.

Our work has focused on the possible effects of Ca^{2+} channels on the chemosensitive response of LC neurons. We have previously reported that hypercapnia activates L-type Ca^{2+} channels in LC neurons [13,20,21]. In LC neurons from young neonates, this activation seems to contribute to the increased firing rate response induced by elevated

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CO_2/H^+ [13]. However, in LC neurons from older neonates the activation of Ca^{2+} channels by hypercapnia stimulates K_{Ca} channels to produce a braking effect on the chemosensitive firing rate response [20]. This effect of hypercapnia on Ca^{2+} channels is unexpected since acidification is commonly expected to inhibit Ca^{2+} channels [36,39]. Recent findings provide evidence that elevated intracellular HCO_3^- is involved in the pathway by which hypercapnia activates the L-type Ca^{2+} current in LC neurons [21]. It was hypothesized that this activation might involve soluble adenylyl cyclase (sAC).

Little is known about a HCO_3^--sensitive mechanism involved in the chemosensitive response of brainstem neurons, but a role for HCO_3^- in the chemosensitive response of peripheral chemoreceptors has been hypothesized [38]. This study determined that the CO_2/H^+ activation of L-type Ca^{2+} channels in glomus cells was blocked by an inhibitor of protein kinase A (PKA) and occurred in association with an intracellular elevation of cAMP [38]. These findings showed that hypercapnia induced elevation of L-type Ca^{2+} channels through activation of PKA. The authors speculated that hypercapnia could activate PKA through a HCO_3^--dependent mechanism. However, a more recent study has shown that the mechanism is dependent on acidosis and does not involve a HCO_3^--dependent mechanism [28].

Soluble adenylyl cyclase (sAC) has been characterized as an intracellular HCO_3^--dependent means of producing cAMP [5,7,25,46]. Since intrinsic chemosensitivity requires that cells respond to CO_2, and that the diffusion of increased CO_2 across cell membranes results in elevated HCO_3^-, it follows that chemosensitive cells expressing sAC could increase their cAMP levels in response to hypercapnia. The presence of sAC in LC neurons has been shown in a preliminary report [27], which raises the possibility that chemosensitive cells of the LC may utilize a sAC-dependent pathway for the activation of their L-type Ca^{2+} channels in response to hypercapnia.

In the current study, we hypothesize that a sAC-dependent pathway is responsible for the CO_2/H^+ activation of the L-type Ca^{2+} current in LC neurons. If so, the addition of dibutyryl-cAMP (db-cAMP) should mimic the hypercapnia-induced increase in Ca^{2+} current [20]. Conversely, we expect that the nominal absence of CO_2/HCO_3^- from the superfusion solution will decrease the sensitivity of this Ca^{2+} current to hypercapnia. In addition, we expect to find the presence of the HCO_3^--dependent sAC enzyme in the cytoplasm of neonatal LC neurons using immunohistochemistry. We have shown that the activation of Ca^{2+} currents in LC neurons from rats older than ~ P10 decreases the firing rate response to hypercapnia via the subsequent activation of large-conductance Ca^{2+}-activated BK channels [20]. If Ca^{2+} channels are activated by hypercapnia through a sAC-mediated mechanism, we further expect that the sAC inhibitors 2-hydroxyestradiol (2HE) or 2-(1H-benzo[d]imidazole-2-ylthio)-*N*'-(5-bromo-2-hydroxybenzylidene) propanehydrazide (KH7) [3,25,33] or the PKA inhibitor H89 will increase the firing rate response to hypercapnia of LC neurons from older neonates to a similar extent as does the BK channel inhibitor paxilline [20] while the transmembrane adenylyl cyclase (tmAC) inhibitor 2',5'-dideoxyadenosine (ddAdo) [3] will have no effect on the firing rate response to hypercapnia of LC neurons from older neonates. Our findings strongly support our hypothesis that a sAC-mediated pathway leads to the activation of L-type Ca^{2+} channels by hypercapnia in LC neurons.

A preliminary report of our findings has previously been published [20].

2. Materials and methods

2.1. Ethical approval

All procedures in which animals were involved were reviewed and approved by the Wright State University Institutional Animal Care and Use Committee and are in agreement with standards set out in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Wright State University is accredited by AAALAC and is covered by NIH Assurance (no. A3632-01).

2.2. Slice preparation

Neonatal Sprague–Dawley rats postnatal (P) age P3–P16 of mixed sex were used in these studies. Depending on the age of the neonate, they were anesthetized using either 100% CO_2 or hypothermia and then decapitated. The brainstem was removed and a vibratome (Pelco Vibratome 1000) was used to make coronal brain slices. Slicing was done in ice-cold (4–6 °C) artificial cerebrospinal fluid (aCSF) solution. Slices of the pons (containing the LC) were maintained in aCSF equilibrated with 5% CO_2/95% O_2 at room temperature until used (1–4 h after slicing). For all experiments, slices were continuously superfused at a rate of ~4 ml/min by gravity flow using solutions held at 35 °C.

2.3. Solutions

All brain slices were immersed in aCSF solution unless indicated otherwise. This solution consisted of (in mM): 124 NaCl, 3 KCl, 1.3 $MgSO_4$, 26 $NaHCO_3$, 1.24 NaH_2PO_4, 10 glucose, and 2.4 $CaCl_2$ and was equilibrated with 5% CO_2/95% O_2, pH ~7.45 (at 35 °C). Hypercapnic solutions had the same composition but were equilibrated with 15% CO_2/85% O_2, pH ~7.0. This level of CO_2 was chosen to maximize the activation of the cellular signaling pathway being studied [17,32]. In nominally CO_2/HCO_3^- free solutions, HEPES buffer isosmotically replaced the HCO_3^- in aCSF and the solution was equilibrated with 100% O_2. The pH of the HEPES aCSF solution was adjusted to 7.45 and 7.0 (similar to the normal aCSF and hypercapnic solutions, respectively) using HCl and NaOH. The whole cell pipette filling solution consisted of (in mM): 130 K-gluconate, 0.4 EGTA, 1 $MgCl_2$, 0.3 GTP, 2 ATP, and 10 HEPES, and was buffered to a pH of ~7.35 using KOH. For intracellular Ca^{2+} (Ca^{2+}_i) measurements, 250 μM of the Ca^{2+}-sensitive fluorescent dye Fura-2 was also added to the pipette solution. The whole cell pipette filling solution for voltage clamp studies of the Ca^{2+} current consisted of (in mM): 130 CsCl, 10 EGTA, 1 $MgCl_2$, 0.3 GTP, 2 ATP, 10 HEPES, and 10 tetraethylammonium (TEA), buffered to pH ~7.45 using CsOH. For immunohistochemistry studies of sAC, the phosphate buffered saline (PBS) solution contained (in mM): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, and 1.47 KH_2PO_4.

2.4. Measurement of intracellular Ca^{2+}

We loaded LC neurons with the Ca^{2+}-sensitive dye Fura-2 (250 μM) from the whole cell patch pipette. Dye-loaded neurons were alternately excited at 340 nm and 380 nm using a Sutter Lamda 10-2 filter wheel (light from a 75 W xenon arc lamp). Emitted fluorescence (505 nm) was intensified by a GenIISys Image intensifier and captured by a CCD camera. Fluorescence images were acquired using a Gateway 2000 E-3100 computer and analyzed with MetaFluor 4.6r software. Images were acquired every 15 s (~2 s acquisition time). Photobleaching was reduced by blocking excitation light between acquisitions. We did not calibrate the Fura-2 fluorescence and instead used arbitrary fluorescence units to monitor increases or decreases in R_{fl}. For analysis, R_{fl} values were estimated by averaging at least 5 values before, during and after db-cAMP administration.

2.5. Electrophysiological recordings

All electrophysiological recordings used in this study were whole cell recordings. Whole cell pipettes were pulled to a tip resistance of ~5 MΩ using thin-walled borosilicate glass (outer diameter 1.5 mm, inner diameter 1.12 mm). We visualized LC neurons with an upright microscope (Nikon Eclipse 6600) using an ×60 water-immersion objective. A visualized neuron was patched, forming a gigaohm seal. Membrane potential (V_m) was measured in both current and voltage clamp