Respiratory Physiology & Neurobiology 187 (2013) 217–223

Contents lists available at SciVerse ScienceDirect

Respiratory Physiology & Neurobiology

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

Stress peptide PACAP stimulates and stabilizes neonatal breathing through distinct mechanisms

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ARTICLE INFO

Article history:
Accepted 8 April 2013

Keywords:
Carotid body
Neuropeptide
Variability
Neonatal apnea
Sudden infant death syndrome (SIDS)

ABSTRACT

Pituitary adenylate cyclase-activating peptide (PACAP) is an important mediator of the stress response and is crucial in maintaining breathing in neonates. Here we investigate the role of exogenously applied PACAP in neonatal breathing using the neonatal rat *in situ* working heart-brainstem preparation. A 1-min bolus of 250 nM PACAP-38 caused an increased in respiratory frequency that was rapid and transient, but had no effect on neural tidal volume or neural minute ventilation. Denervation of the carotid body abolished this effect. PACAP had a persistent effect on breathing stability in both carotid body-intact and -denervated preparations, as shown by decreases in respiratory variability 5 min following application. These data suggest that PACAP released during stress acts via carotid body dependent and independent mechanisms to stimulate and stabilize breathing. These mechanisms may account for PACAP's critical role in defending neonatal breathing against environmental stress.

1. Introduction

Stress during early life can have long-term physiological effects that can continue into adulthood. Thus, the study of neonatal stress is important for understanding both the pathologies that may arise during the neonatal period, and the long-lasting physiological consequences that manifest in the adult. The respiratory system is not immune to the consequence of neonatal stress; as illustrated recently, neonatal rats separated from their mothers or exposed to intermittent hypoxia shortly after birth exhibit an augmented hypoxic response that remains abnormal into adulthood (Genest et al., 2004; Pawar et al., 2008). However, how neonatal stress affects the respiratory system is poorly understood.

In adults, the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) has been identified as a crucial mediator of most stress responses (Dow et al., 1994; Grinevich et al., 1997; Hannibal et al., 1995; Stroth and Eiden, 2010). For example, in the HPA axis, approximately 50% of PACAP-expressing neurons in the parvocellular region of the paraventricular nucleus (PVN) co-express corticotropin-releasing hormone (CRH) (Hannibal et al., 1995), intravenous administration of PACAP causes an increase in the expression of CRH in these neurons (Grinevich et al., 1997), and the induction of CRH mRNA in PACAP-null mice is diminished in the PVN following restraint stress, as is the secretion of corticosterone from the adrenal cortex, as compared to wildtype mice (Stroth and Eiden, 2010; Stroth et al., 2011). PACAP also plays a crucial role in the sympathoadrenal stress pathway. In this pathway, PACAP is critical for the biosynthesis and release of catecholamines from the adrenal medulla (Hamelink et al., 2002; Haycock, 1996; Stroth and Eiden, 2010).

Previously, we demonstrated PACAP plays a critical role in regulating neonatal breathing during times of stress (Cummings et al., 2008, 2004). PACAP-null mice survive to weaning when raised at an ambient temperature of 24 °C but are prone to a sudden death phenotype when raised at a slightly cooler (19 °C) temperature (Gray et al., 2002). Studies using whole-body plethysmography have demonstrated that PACAP-null neonatal mice have lower baseline ventilation than their wildtype littermates when exposed to cold stress and have blunted ventilatory responses to hypoxia and hypercapnia that may be due to defects in the respiratory rhythm generator (Arata et al., 2013; Cummings et al., 2008, 2004). Such findings indicate that PACAP is important in protecting neonatal breathing against environmental and respiratory stress, though the precise mechanism has not been elucidated.

The primary sensors for respiratory stress are the peripheral respiratory chemoreceptors, the carotid bodies. Data from adult dogs suggest that PACAP strongly stimulates these organs (Runcie et al., 1995), and immunohistochemical studies have shown that PACAP and at least one of its receptors are present in the adult rat carotid body (Lam et al., 2012). However, PACAP and receptors for PACAP are highly expressed throughout the central and peripheral nervous system, including several respiratory-related nuclei (Hannibal, 2002), with one study suggesting that PACAP is

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http://dx.doi.org/10.1016/j.resp.2013.04.009

capable of increasing hypoglossal activity in the preBötzinger slice preparation (Peña, 2010). In addition, PACAP stimulates the adrenal glands, which are thought to function as oxygen sensors in neonates and secrete catecholamines in response to sympathetic activation caused by systemic hypoxia in mammals of all ages (Rico et al., 2005; Seidler and Slotkin, 1985, 1986; Thompson et al., 1997). Any of these sites may be the point at which PACAP plays a critical role in protecting neonatal breathing against stress.

The widespread distribution of PACAP receptors within the CNS makes it possible that PACAP has a central effect. However, given the powerful action of PACAP on the adult carotid body, we hypothesize that PACAP's stimulatory effect on neonatal respiration is mediated peripherally by the carotid bodies. To test this hypothesis we used the *in situ* rat working heart-brainstem preparation modified for neonates (Dutschmann et al., 2000). The neonatal working heart-brainstem preparation allows the control of breathing to be studied in a non-anesthetized preparation, free from descending, hormonal and adrenal influences. Unlike isolated and superfused neonatal rodent brainstem preparations, the brainstem of the neonatal working heart-brainstem preparation is oxygenated throughout and the peripheral chemoreceptors are intact (Dutschmann et al., 2000). It thus offers many of the advantages of reduced preparations, yet allows the study of chemosensitivity without the confounding influence of brainstem anoxia.

2. Methods

Experimental procedures conform to Canadian Council for Animal Care guidelines and were approved by the University of Calgary Animal Care Committee. Female Sprague-Dawley rats with litters were obtained from Charles River Laboratories, Quebec, Canada, and housed in the University of Calgary's Health Sciences Animal Resource Center on a 12:12 light/dark cycle. Pups were used for experiments between postnatal days 4-7 (P4-P7). Animals were provided with food and water *ad libitum*. A total of 42 animals were used for this study, divided into 4 experimental groups.

2.1. Neonatal working heart-brainstem preparation

For these experiments we used the neonatal working heart-brainstem preparation (Dutschmann et al., 2000). We have added a number of additional modifications that increase the number of viable preparations. Preparation of the neonatal working-heart-brainstem was performed as follows:P4-P7 rats were given halothane until unresponsive to foot pinch. Pups were then bathed in ice-cold perfusate (1 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 4 mM KCl, 24 mM $NaHCO_3$, 115 nM NaCl, 10 mM glucose, 12 mM sucrose, 2 mM $CaCl_2$) and transected caudal to the diaphragm. Next, pups were decerebrated and pinned to a Sylgard® dish ventral side up. The chest was skinned and the ribcage cut back to expose the heart. A small hole was cut in the left ventricle of the heart through which a cannula was inserted and glued in place using a cyanoacrylate-based adhesive. A pump transferred perfusate from a large container (into which 40 Torr PCO_2 and a balance of oxygen was continuously bubbled), through a heat exchanger (set to deliver perfusate to the preparation at 34 °C), bubble trap, and filters (45 μm and 25 μm pore sizes) into the heart. The descending aorta was glued shut to help maintain fluid pressure in the preparation. Perfusate exiting cut blood vessels was drained back into the perfusate vessel via gravity and eventually re-circulated. While the working heart-brainstem preparation in the adult rat uses a double-lumen catheter with a pressure transducer to measure the perfusion pressure within the aorta (Day and Wilson, 2005) and the adult mouse preparation uses a hypodermic needle inserted into the heart to monitor pressure (Paton, 1996), the neonatal preparation is too small to measure aortic pressure in the same way. As a result, when the neonatal working heart-brainstem preparation was first developed, eupnic-like respiratory discharge was used to indicate adequate perfusion pressure for the preparation (Paton, 1996). With our modified approach, in addition to monitoring adequacy of perfusion pressure via respiratory output, we also used a vertical column to govern the pressure of perfusate entering the preparation. Specifically, a 120 cm glass column was added to the perfusion circuit to produce a maximum pressure of 90 mm Hg when the perfusate enters the preparation (the same pressure used for the *in situ* preparation in adult rats as measured by a pressure transducers). This pressure produces ramping phrenic (eupnic-like) output in most P4–P7 rats. Upon initiation of breathing following cannulation, preparations were paralyzed using rocuronium bromide (Sigma–Aldrich) (approximate dose of 0.04 μg/L) to aid instrumentation. In carotid body denervated preparation, denervations were performed by scraping out the bifurcation of the common carotid artery with sharp forceps. Silver electrodes were then hooked up to the phrenic nerve (signal electrode) and diaphragm (reference electrode) to record inspiratory activity. The signal from the electrodes was amplified (Brownlee Precision Model 440 & A-M Systems Differential AC Amplifier Model 1700), filtered (low cut off at 150 Hz, high cut off at 500 Hz), rectified, integrated (time constant of 200 ms), digitized (Digidata 1322A, Axon Instruments), and stored using Axon Axoscope software (Molecular Devices) at a sampling rate of 1 kHz.

2.2. Protocol

Four experimental groups were used. In two of these groups the carotid bodies were left intact and in the other two groups the carotid bodies were denervated. Following a 30-min period of stabilization, all preparations received a 1 mL 60-s bolus of gas-equilibrated perfusate injected into the line upstream of the heat exchanger. In one group with carotid bodies intact (the carotid body-intact PACAP group; $n = 10$) and one group with carotid bodies denervated (the carotid body-denervated PACAP group $n = 12$), the bolus contained 6.25 μM PACAP-38 (AnaSpec). The concentration of PACAP that reached the preparation was 250 nM. The remaining groups where given a bolus containing vehicle (perfusate only; carotid body-intact vehicle group: $n = 10$; carotid body-denervated vehicle group: $n = 10$). The experimenter was blinded to whether preparations were receiving PACAP or vehicle. Bolus administration was followed by a washout period.

2.3. Analysis

Recordings were analyzed offline using Phrenic Peak Detector software (Chinook Technologies, version 63) and TPVAnalysis software (Fishman et al., 2012). To study the effect of PACAP on the control of breathing we quantified phrenic activity in the 300 s prior to bolus administration (baseline period), and 0–30 s (immediate effect) and 300–330 s (latent effect) following bolus administration. Respiratory parameters were normalized to baseline values for each preparation. SPSS version 19 was used to conduct statistical analysis. One-way ANOVAs with Tukey post-hocs were performed to test differences in respiratory frequency (fr), neural tidal volume (nVt), and neural minute ventilation (nVe) in the immediate and latent timeframes between groups (i.e. carotid body-intact vehicle, carotid body-intact PACAP, carotid body-denervated vehicle, and carotid body-denervated PACAP groups). Breath-by-breath analysis was performed to determine whether PACAP had any effect on breathing variability. Independent samples *t*-tests with Bonferroni correction were performed on data for short- and long-term variability measures. All data are represented as mean ± standard