Activity of Cardiorespiratory Networks Revealed by Transsynaptic Virus Expressing GFP

MUSTAPHA IRNATEN,1 ROBERT A. NEFF,1 JIJIANG WANG,1 ARTHUR D. LOEWY,2 THOMAS C. METTENLEITER,3 AND DAVID MENDELOWITZ1

1Department of Pharmacology, George Washington University, Washington, DC 20037; 2Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110; and 3Federal Research Centre for Virus Diseases of Animals, Institute of Molecular Biology, Friedrich-Loeffler Institutes, D-17498 Insel Riems, Germany

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INTRODUCTION

Heart rate is determined primarily by the centrally mediated tonic and reflex activity of parasympathetic outflow to the heart (Heymans and Neil 1958; Spyer 1981). Cardiac vagal activity is diminished and unresponsive in many disease states, and a delay in the inhibitory actions of this autonomic motor system following exercise is a powerful predictor of overall mortality (Cole et al. 1999; Eckberg et al. 1971; Larovere et al. 1988; Vanoli et al. 1991). The control of heart rate is also intertwined with the generation of respiratory rhythm within the CNS (Anrep et al. 1935; Gilbey et al. 1984). In each respiratory cycle, the heart beats more rapidly in inspiration and slows during postinspiration and expiration (referred to as respiratory sinus arrhythmia) (Anrep et al. 1935). The degree of respiratory sinus arrhythmia is often a clinical indicator of cardiorespiratory function, particularly during the neonatal and postnatal period (Hathorn 1978; Hrushesky 1991). Despite the clinical importance of respiratory modulation of heart rate, the neuronal circuit for this cardiorespiratory interaction has not yet been identified. One possible source of cardiorespiratory interactions may involve superior laryngeal neurons that are active in respiration and are colocalized with cardiac vagal neurons in the nucleus ambiguus. Here we demonstrate that superior laryngeal motorneurons project to cardiovagal neurons. The Bartha strain of pseudorabies virus (PRV), an attenuated swine alphaherpesvirus, can be used as a transsynaptic marker of neural circuits. Bartha PRV invades neuronal networks in the CNS through peripherally projecting axons, replicates in these parent neurons, and then travels transsynaptically to continue labeling the second- and higher-order neurons in a time-dependent manner. A Bartha PRV mutant that expresses green fluorescent protein (GFP) was used to visualize and record from neurons that determine the vagal motor outflow to the heart. Here we show that Bartha PRV-GFP-labeled neurons retain their normal electrophysiological properties and that the labeled baroreflex pathways that control heart rate are unaltered by the virus. This novel transsynaptic virus permits in vitro studies of identified neurons within functionally defined neuronal systems including networks that mediate cardiovascular and respiratory function and interactions. We also demonstrate superior laryngeal motoneurons fire spontaneously and synapse on cardiac vagal neurons in the nucleus ambiguus. This cardiorespiratory pathway provides a neural basis of respiratory sinus arrhythmias.

METHODS

Retrograde transneuronal virus and fluorescent labeling

The gene encoding enhanced GFP, a bioluminescent protein of 238 amino acids originally isolated from the jellyfish Aequorea victoria, was inserted in the nonessential glycoprotein G (gG) gene of the transneuronal viral tracer Bartha-PRV. The coding sequence was cloned in frame behind the first seven codons of the gG gene under control of the strong gG promoter in the Bartha-PRV. To label cardiovagal neurons and the neurons that project to them, 0.5–20 μL of virus [titer = 10^8 plaque forming units per milliliter (pfu/ml)] was injected into the pericardial sac of methoxyflurane anesthetized Sprague Dawley rats (p4–p13) of either sex. In control experiments, injections of Bartha-PRV-GFP into the chest cavity, but outside the pericardial sac, did not produce any labeling in the brain stem. In immunohistochemical and electrophysiological experiments, a bilateral stellectomy was performed to remove the sympathetic innervation.
to the heart (Pardini et al. 1989). To independently identify superior laryngeal motorneurons and cardiovagal neurons, crystals of the fluorescent tracer tetramethylrhodamine isothiocyanate perchlorate (DiI) were placed onto the superior laryngeal nerve and rhodamine (1% solution XRTIC, 20–50 μL) was injected into the pericardial sac.

**Immunohistochemistry procedures**

In immunohistochemistry studies, the animals were anesthetized with methoxyflurane and perfused through the heart with 0.9% saline, followed by 4% paraformaldehyde, stored for 12–24 h, and then the brain stem was cut into 30-μm-thick sections using a cryostat. Tissue sections were incubated for 1 h in 0.1 M PBS, 2% bovine serum albumin (BSA), and 0.3% Triton X-100, washed, and incubated overnight at 4°C in a 1:1000 dilution of GFP monoclonal antibody (Clontech), followed by a 1:100 dilution of goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate secondary antibody (Sigma). Specificity was determined by omitting the primary antibody from the incubation or by omitting the secondary antibody. Slices that included the nucleus ambiguus were imaged with a Bio-Rad (Her-cules, CA) MRC-1000 confocal microscope.

**Electrophysiological and in vivo studies**

For electrophysiological experiments, the animals were anesthetized with methoxyflurane and killed by cervical dislocation, and the brain was sectioned into 250-μm sections. Neurons were imaged with infrared and fluorescent illumination. Standard patch-clamp electrophysiological techniques were used while the slices were continuously perfused (2–3 ml/min) with a perfusate of the following composition (in mM): 125 NaCl, 3 KCl, 2 CaCl₂, 26 NaHCO₃, 5 dextrose, and 5 HEPES, constantly bubbled with 95% O₂-5% CO₂, and maintained at pH 7.4. Patch pipettes were filled with a solution consisting of (in mM): 130 KGlutamate, 10 HEPES, 10 EGTA, 1 CaCl₂, and 1 MgCl₂, in perforate patch experiments nystatin (258 U/ml) was included. Action potentials were recorded using the perforated-patch access and current-clamp configuration. Voltage-gated ionic currents were studied using the voltage-clamp configuration. Studies of the baroreceptor reflex were conducted in age-matched animals 10–12 days old (anesthetized with urethan, 1.3 mg/kg, IP) using standard phenylephrine infusions to increase blood pressure and evoke the baroreflex decrease in heart rate. The baroreflex curves were generated (Origin 5.0) using a sigmoidal logistic fit, y = A₂ + (A₁ − A₂)[1 + (x/x₀)p]. Two animals had a paradoxical increase in heart rate and were omitted from analysis. A paradoxical response in rats this age has also been observed by others (Kasparov and Paton 1997). All experiments were performed in compliance with institutional guidelines at George Washington University.

**RESULTS**

To identify cardiovagal neurons and in particular the neur-ons that synapse on them, we first performed a series of experiments in which Bartha PRV-GFP was injected into the pericardial sac of Sprague-Dawley rats with increasing survival periods. After two days of survival, only cardiovagal neurons were labeled in the nucleus ambiguus (Fig. 1). The labeling pattern was identical to the population of cardiovagal neurons labeled with conventional retrograde fluorescent tracers such as rhodamine (XRTIC) (Fig. 1). To label superior laryngeal moto-neurons, the fluorescent tracer DiI was applied to the super-iior laryngeal nerve 2 days prior to death. Superior laryngeal motorneurons were in close proximity to cardiovagal neurons labeled 2 days after injection of Bartha-PRV-GFP into the pericardial sac, but no superior laryngeal motorneurons con-tained the virus (Fig. 1).

**FIG. 1.** Cardiovagal neurons were labeled with Bartha pseudorabies virus-green fluorescent protein (PRV-GFP) 2 days after injection of the virus into the pericardial sac. A: Bartha-PRV-GFP (green) was present only in cardiovagal neurons identified with the traditional fluorescent rhodamine tracer XRTIC (red). All cardiovagal neurons contained both virus and rhodamine (yellow, n = 6). B: superior laryngeal motorneurons labeled with the fluorescent tracer DiI (red) were in close proximity to, but were not colocalized in, neurons labeled with the Bartha-PRV-GFP (green) 2 days after injection of the virus into the pericardial sac (n = 7). Scale bar is 25 μm.

The utility of Bartha-PRV-GFP as a transneuronal tracer in living tissue was examined electrophysiologically 2 days after virus injection into the pericardial sac. Cardiovagal neurons could be easily visualized by both a traditional fluorescent tracer (XRTIC) and the GFP fluorescent signal in an in vitro 250-μm slice of tissue 2 days after injection of the virus and XRTIC into the pericardial sac (Fig. 2). The electrophysiologi-cal properties of both XRTIC-labeled and PRV-infected car-diovagal neurons were analyzed by patch-clamp recordings. The voltage-gated currents and firing properties in Bartha PRV-GFP or XRTIC-labeled cardiovagal neurons were indistinguishable. Cardiovagal neurons were silent with a typical resting membrane potential of −70 mV. On injection of depolar-izing current cardiovagal neurons had a pattern of action potential firing with little delayed excitation or spike frequency adaptation (Fig. 2). Depolarizations to potentials more positive than −50 mV evoked a rapidly activating and inactivating sodium current that has been shown previously to be tetrodo-toxin resistant, requiring 1 μM tetrodotoxin for complete block (Mendelowitz 1996, 1999). Immediately following the sodium current transient outward and long-lasting potassium currents were elicited which consist of an A-type and a delayed rectifier potassium current, respectively (Mendelowitz 1996, 1999).

After establishing that Bartha PRV-GFP could be used to identify neurons in specific functional circuits for subsequent electrophysiological experiments, a second series of immuno-histochemical experiments was performed in which animals were killed 3 days after injection of virus into the pericardial sac. In addition DiI was applied to the superior laryngeal nerve 3 days prior to sacrifice. After 3 days, the labeling was still present in cardiovagal neurons and advanced to other neurons in the nucleus ambiguus as well as additional brainstem regions including the nearby periamigdual area and the nucleus tractus solitarius. Within the nucleus ambiguus, superior laryngeal motorneurons were colabeled with both DiI and Bartha-PRV-GFP, demonstrating that they innervate cardiovagal neurons (Fig. 3). Superior laryngeal motorneurons transsynaptically...
labeled 3 days after injection of Bartha-PRV-GFP into the pericardial sac could also be readily visualized by GFP fluorescence in an in vitro slice of tissue (Fig. 3). The traditional retrograde tracer DiI also identified these neurons. Superior laryngeal motorneurons typically fire spontaneously and continuously at a frequency of 5–7 Hz. The firing properties and individual action potentials in Bartha PRV-GFP- or DiI-labeled superior laryngeal motorneurons were identical.

Since the electrophysiological studies indicate both cardiac vagal neurons and superior laryngeal motorneurons targeted by the virus were electrophysiologically indistinguishable from these neurons identified with traditional tracers, we examined whether the baroreceptor reflex was altered in animals 3 days after injection of the virus into the pericardial sac. Phenylephrine was infused to increase blood pressure and evoke baroreflex-mediated decreases in heart rate. The baroreflex responses in animals labeled with the virus (left, n = 11) were identical to the properties of cardiac vagal neurons labeled with baroreflex activity has been implicated in diseases such as cardiac arrhythmia (Spyer 1981). Respiratory sinus arrhythmia is present in healthy fetuses, newborns, and mature animals and humans (Elghozi et al. 1991). However, in distressed

**DISCUSSION**

It is well known that the neuronal projections from the brain to the heart strongly influence cardiac function, and an abnormal cardiorespiratory network reveals cardiorespiratory networks...
fetuses, as well as partially asphyxiated newborns, a slowing of the heart rate and diminished respiratory sinus arrhythmia is strongly correlated with low postnatal Apgar scores (a quick clinical assessment of overall newborn well being) and subsequent neonatal mortality such as in Sudden Infant Death syndrome (Meny et al. 1994; Schechtman et al. 1992). This study, using the Bartha-PRV-GFP transneuronal retrograde tracer has identified a pathway that can mediate the normal interactions between the respiratory system and the control of cardiac function that may be altered in diseases of the cardiorespiratory systems. The projection from superior laryngeal motorneurons to cardiovascular neurons may be responsible for respiratory modulation of heart rate. The nonbursting spontaneous activity in superior laryngeal neurons is unlikely to be responsible for generating respiratory rhythms but is more likely involved in coordinating motor activity to the heart and respiratory muscles. Superior laryngeal neurons may provide an excitatory input to cardiovascular neurons during postinspiration. In addition to this pathway, other work has shown cardiovascular neurons are inhibited by a GABAergic input during inspiration (Gilbey et al. 1984). The use of the PRV-GFP transneuronal retrograde tracer may provide an opportunity to study this and other pathways that are also involved in cardiorespiratory interactions.

A very recent report has examined the utility of using a PRV that also expresses enhanced GFP in an in vitro study of retinal pathways. Transsynaptically labeled neurons in the suprachiasmatic nucleus and retinal ganglia retained their major excitatory and inhibitory inputs, suggesting this virus does not interfere with the synaptic function in retinal pathways (Smith et al. 2000). This study, however, did not determine whether PRV-GFP caused any changes in firing properties or voltage-gated currents in the labeled neurons or evoked any functional changes in the retinal pathways. The present work demonstrates that the firing properties and voltage-gated currents in PRV-GFP-labeled cardiorespiratory neurons are unaltered by PRV-GFP. Moreover, this study identifies a previously undescribed synaptic pathway from superior laryngeal neurons to cardiovascular neurons that may be involved in mediating cardiorespiratory rhythms.

The present work, and that of Smith et al. (2000), indicates fluorescent viral transneuronal tracers, such as Bartha PRV-GFP are useful tools for in vitro physiological studies, particularly since in the early phases of the infection (in these studies 2–3 days), the electrophysiological properties of the neurons appear normal. In addition, the synaptic input to neurons and the reflex control of the labeled pathways are not altered by these viruses. It is anticipated that this virus can be used in the cardiorespiratory system to identify other neurons that synapse on cardiovascular neurons and neurons involved in other cardiorespiratory functions, which can then be studied electrophysiologically. It is also likely this virus can be utilized to introduce gene products other than GFP to deliberately alter the function of specific neurons in CNS pathways that control cardiorespiratory function.

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