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CHARACTERIZATION OF GLUTAMATERGIC NEURONS IN THE RAT ATRIAL INTRINSIC CARDIAC GANGLIA THAT PROJECT TO THE CARDIAC VENTRICULAR WALL

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Abstract—The intrinsic cardiac nervous system modulates cardiac function by acting as an integration site for regulating autonomic efferent cardiac output. This intrinsic system is proposed to be composed of a short cardio-cardiac feedback control loop within the cardiac innervation hierarchy. For example, electrophysiological studies have postulated the presence of sensory neurons in intrinsic cardiac ganglia (ICG) for regional cardiac control. There is still a knowledge gap, however, about the anatomical location and neurochemical phenotype of sensory neurons inside ICG. In the present study, rat ICG neurons were characterized neurochemically with immunohistochemistry using glutamatergic markers: vesicular glutamate transporters 1 and 2 (VGLUT1; VGLUT2), and glutaminase (GLS), the enzyme essential for glutamate production. Glutamatergic neurons (VGLUT1/VGLUT2/GLS) in the ICG that have axons to the ventricles were identified by retrograde tracing of wheat germ agglutinin–horseradish peroxidase (WGA–HRP) injected in the ventricular wall. Co-labeling of VGLUT1, VGLUT2, and GLS with the vesicular acetylcholine transporter (VAChT) was used to evaluate the relationship between post-ganglionic autonomic neurons and glutamatergic neurons. Sequential labeling of VGLUT1 and VGLUT2 in adjacent tissue sections was used to evaluate the co-localization of VGLUT1 and VGLUT2 in ICG neurons. Our studies yielded the following results: (1) ICG contain glutamatergic neurons with GLS for glutamate production and VGLUT1 and 2 for transport of glutamate into synaptic vesicles; (2) atrial ICG contain neurons that project to ventricle walls and these neurons are glutamatergic; (3) many glutamatergic ICG neurons also were cholinergic, expressing VAChT; (4) VGLUT1 and VGLUT2 co-localization occurred in ICG neurons with variation of their protein expression level. Investigation of both glutamatergic and cholinergic ICG neurons could help in better understanding the function of the intrinsic cardiac nervous system.

Key words: intrinsic cardiac ganglia, glutaminase, vesicular glutamate transporter 1 & 2, vesicular acetylcholine transporter, wheat germ agglutinin–horseradish peroxidase, sensory neurons.

INTRODUCTION

The intrinsic cardiac ganglia (ICG) consist of groups of ganglion neurons that reside on the dorsal surface of the atrium. This ganglionic system is considered to be composed of a short cardio-cardiac neuronal feedback loop that functions in modulating regional cardiodynamics. Within this system, three major neuronal components have been proposed: autonomic (parasympathetic and sympathetic) efferent neurons, local circuit neurons, and sensory neurons (Armour, 1999, 2004; Kukanova and Mravec, 2006; Armour, 2008). The autonomic efferent neurons within the ICG receive sympathetic and parasympathetic preganglionic information from higher neuronal hierarchy to modulate heart rate, conduction velocity and contractility (Ardell et al., 1988; Gatti et al., 1995; Cheng et al., 1999; Cheng and Powley, 2000; Armour, 2008). Efferent neurons project to cardiac tissue including the sinoatrial (SA) node, atrioventricular (AV) node and contractile tissues (e.g., papillary muscle). Although often thought of as relay ganglia, the ICG also were proposed to contain both interneurons and sensory neurons. Local mechanical and chemical stimuli on the cardiac ventricles and major vessels activate ICG neurons and the information is transmitted to local circuit neurons and/or autonomic efferent neurons for adjusting their neuronal activity (Ardell et al., 1991; Armour et al., 1997; Thompson et al., 2000; Armour, 2008).

In earlier electrophysiology studies, ‘‘sensory-like” cells were described in the ICG of different species and were characterized by prolonged after hyperpolarization (AHP) and inward rectification at slightly negative membrane potentials. These membrane electrical properties resemble the electrophysiology pattern of primary sensory neurons in dorsal root ganglia (DRG) (Selyanko, 1992; Edwards et al., 1995). In histology studies, the presence of bipolar or pseudounipolar neurons in

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Abbreviations: BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DRG, dorsal root ganglia; GLS, glutaminase; HRP, horseradish peroxidase; ICG, intrinsic cardiac ganglia; iGluRs, ionotropic glutamate receptors; MGI, mean gray intensity; mGluRs, metabotropic glutamate 1 receptors; NG, nodose ganglion; PBS, phosphate-buffered saline; PVP, polyvinylpyrolidone; TB, Toluidine Blue; TG, trigeminal ganglion; VAChT, vesicular acetylcholine transporter; VGLUTs, vesicular glutamate transporters; WGA, wheat germ agglutinin.

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ICG resembles primary sensory neurons in DRG and nodose ganglion (NG) (Pauza et al., 1997; Moravec and Moravec, 1998). Moreover, ventricular epicardial mechanical stimulation induces neuronal activation in ICG (Horackova et al., 1999; Thompson et al., 2000). Chemicals, such as substance P, ATP, neuropeptide Y, bradykinin and adenosine, change the neuronal activity within the ICG when applied to the ventricular cardiac surface (Armour et al., 1998; Armour, 1999; Thompson et al., 2000). These studies indicate that some ICG neurons possess sensory properties and are capable of transmitting information about regional cardiac function and milieu. An understanding, however, of the neurochemical phenotype of these potential sensory neurons and their anatomical location inside the ICG is still lacking.

Neurochemical and electrophysiological studies have determined that DRG primary sensory neurons have a glutamatergic neurotransmitter phenotype (Miller et al., 2011). In the current study, we propose that ICG primary sensory neurons use glutamate as their neurotransmitter. In order to evaluate this proposal, we first investigated if glutamatergic neurons were present within ICG using immunohistochemistry for glutaminase (GLS), synthetic enzyme for glutamate, and vesicular glutamate transporter 1 and 2 (VGLUT1 and 2). Secondly, WGA–HRP retrograde tracing from the ventricular wall was used coupled with immunohistochemistry for GLS and VGLUT 1, 2 to detect potential sensory ICG neurons. Thirdly, to compare ICG neurons with glutamatergic vs. cholinergic phenotype, we co-stained with immunohistochemistry for vesicular acetylcholine transporter (VAChT) and glutamatergic neuronal markers, VGLUT1 and 2 and GLS. Fourthly, the potential for simultaneous expression of VGLUT1 and 2 was explored by examination of adjacent serial sections of ICG alternatively stained for VGLUT1 and 2.

EXPERIMENTAL PROCEDURES

Adult Sprague–Dawley (SD) rats (n = 30, 250–350 g) were housed in a 12-h light: 12-h dark cycle and given free access to food and water. All procedures were conducted according to guidelines from the National Institutes of Health (NIH Publications No. 80-23) and were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee (IACUC #2014-02). All efforts were made to minimize the number of animals used and their suffering.

Surgery

In the retrograde tracing study, SD rats (n = 11) were anesthetized with 5% isoflurane and maintained by 3% isoflurane. The depth of anesthesia was tested by the absence of response to pinch of rat hind paw and tail end. To avoid thoracotomy, a trans-diaphragm injection method was chosen. Anesthetized rats were laid supine and the ventral abdominal surgical field was prepared by shaving the hair and cleaning with providone–iodine. A midline incision was made through the abdominal wall below the xiphoid process exposing the abdominal cavity. The liver was retracted, the xiphoid process was lifted, and the heart was observed through the central tendon of diaphragm. Using a 10-μl microinjection syringe, 2–3 μl of WGA–HRP (Vector Laboratories, Inc., CA, USA) was injected trans-diaphragmatically into the cardiac ventricular muscle at an angle between 0° and 15°. A suture knot was placed 1.5–2 mm from the tip of injection needle to maintain a consistent depth of injection into the ventricle. Two–three injections were made into the ventricular wall with a total amount of approximately 8 μl of WGA–HRP injected into the ventricular wall. After injections, both the abdominal muscle and skin were sutured. Animals were allowed to recover from the surgery on a warm cloth and were placed in single cages for 72–84 h. WGA–HRP is transferred to the neuronal cell soma by receptor-mediated internalization and active retrograde transport of endosomes (Aschoff and Hollander, 1982; Kobbert et al., 2000; Hoover et al., 2008).

Tissue preparation

Naïve adult rats (n = 13) of both sex and rats from the retrograde tracer study (n = 11) were intra-peritoneally (i.p.) injected with 2.5% Avertin (3 ml) in phosphate-buffered saline (PBS) for anesthesia and a subsequent i.p. injection of 1-ml xylazine for euthanasia. A thoracotomy was performed to expose the heart and rats were transcardially perfused with 100-ml calcium free Tyrodes solution followed by 300 ml of 0.2% (w/v) paraformaldehyde, 0.8% (w/v) picric acid solution in 0.1 M sodium phosphate buffer. After perfusion, atrial tissue with epicardial fat pad was removed for immunohistochemical evaluation. For absorption control studies, the colon, cornea, skin, sciatic nerve, DRG, trigeminal ganglia (TG), and spinal cord from several rats were removed, also. Tissues were placed in post-fixative for 4 h at 4 °C and stored in 10% sucrose (w/v) at 4 °C until immunohistochemical processing. The atrial tissue and other tissues were laid flat in embedding molds, covered with Shandon M-1 embedding matrix (Thermo Scientific, MI, USA) and frozen in a −20 °C cryostat chamber. Tissues were cut as 16–20-μm frozen sections with a cryostat (Leica CM1850, Leica Biosystems Nussloch GmbH, Germany). Tissues were mounted on gelatin coated Super Frost slides (Fisher Scientific, Pittsburgh, PA, USA) and dried at 38 °C on a slide warmer for 90 min. After this step, sections were rinsed three times with PBS. To identify ICG neurons in the atrial tissue, some sections were stained tinctorially with a Toluidine Blue (TB) working solution (10% TB in 1% sodium chloride, pH = 2.5) for 90 s. Once sections with ICG neurons were identified with light microscopy for TB staining, neighboring slides were selected for immunohistochemistry.

Immunohistochemistry

Atrial tissue sections from naïve rats (n = 13) were incubated in mouse anti-peripherin (Millipore/Chemicon, Billerica, MA, USA) and one of the following primary antibodies: rabbit anti-GLS (1:20,000, gift from Dr. N.