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Research article

Innervation of sinoatrial nodal cells in the rabbit

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ABSTRACT

In spite of the fact that the rabbit is being widely used as a laboratory animal in experimental neurocardiology, neural control of SAN cells in the rabbit heart has been insufficiently examined thus far. This study analyzes the distribution of SAN cells and their innervation pattern employing fluorescent immunohistochemistry on rabbit whole mount atrial preparations. A dense network of adrenergic (positive for TH), cholinergic (positive for ChAT), nitrergic (positive for nNOS) and possibly sensory (positive for SP) NFs together with numerous neuronal somata were identified on the RRCV where the main mass of SAN cells positive for HCN4 were distributed as well. In general, the area occupied by SAN cells comprised nearly the entire RRCV and possessed a three to four times denser network of NFs compared with adjacent atrial walls. Adrenergic NFs predominated noticeably in-between SAN cells. Solitary neuronal somata or somata gathered into small clusters were positive solely for ChAT or nNOS, respectively or simultaneously for both neuronal markers (ChAT and nNOS). Neuronal somata positive for nNOS were more frequent than those positive for ChAT. In conclusion, findings of the present study demonstrate a dense and complex ganglionated neural network of both autonomic and sensory NFs, closely related to SAN cells which spread widely on the RRCV and extend as sleeves of these cells toward the walls of the rabbit RA.

1. Introduction

SAN cells send electrical signals to heart muscle, initiating its contraction. These cells have been an object of investigation in many pathological cases as SAN cells may be a trigger of sudden cardiac death (Christoffels and Moorman, 2009). Employing histological and immunohistochemical techniques, specialized cardiac cells of different functions were observed in the SAN area: pacemaker, transitional myocardial and insulation cells (Atkinson et al., 2013; Bharati and Levine, 1991; Liu et al., 2007; Tellez et al., 2006). HCN4 has been considered as a specific protein of pacemaker cells, allowing a reliable morphological identification of SAN cells (Atkinson et al., 2013; Liu et al., 2007; Tellez et al., 2006).

Physiological studies have shown that acetylcholine and noradrenaline can modulate the rate of SAN myocytes and action potential duration of atrial myocytes over a wide range (Verkerk et al., 2012). This effect may be explained by G protein regulation that suppresses muscarinic sensitivity and parasympathetic tone. It may prove to be a target for the treatment of SAN bradycardia or tachycardia (Verkerk et al., 2012). Moreover, stimulation of postganglionic NFs results in complex changes of pacemaker excitability in SAN cells (Fedorov et al., 2006). Such physiological findings correspond to neuroanatomical observations that the SAN possesses a considerably higher density of NFs relative to the adjacent myocardium (Crick et al., 1999, 1996, 1994; Pauza et al., 2013, 2014a,b; Roberts et al., 1989; Roberts, 1991a) and that two RA neural subplexuses (the dorsal and ventral ones) supply the SAN neural network in various mammalian species and the human (Batulevicius et al., 2005, 2003; Brack, 2014; Pauza et al., 2000, 1999; Rysevaite et al., 2011; Saburkina et al., 2014, 2010). Densities of cholinergic, adrenergic, and peptidergic (sensory) NFs differ significantly among the animal species (Chow et al., 2001; Crick et al., 1999, 1996, 1994). Many previous studies aiming to identify the distribution of nerve fibers in the heart were based on histochemical staining for acetylcholinesterase (AChE) which was considered a trustworthy marker of cholinergic nerve fibers (Crick et al., 1994, 1996, 1999; Chow et al., 2001). However, AChE was also abundantly identified among sensory and sympathetic nerve fibers (Koelle, 1955; Saburkina et al., 2010; Pauza et al., 2014a,b) and, therefore, AChE histochemical staining was altered to an

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; HCN4, hyperpolarization activated cyclic nucleotide-gated potassium channel 4; NFs, nerve fibers; nNOS, neuronal oxide synthase; RA, right atrial or right atrium; RRCV, root of the right cranial vein (superior vena cava); SAN, sinoatrial nodal or sinoatrial node; SP, substance P; TH, tyrosine hydroxylase.

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Table 1
Primary and secondary antisera used in the study.

Antigens	Host	Dilution	Company	Catalog number
Primary				
CHAT	Goat	1:100	Chemicon[a]	AB144P
TH	Mouse	1:400	Chemicon[a]	MAB318
TH	Sheep	1:800	Chemicon[a]	AB1542
CGRP	Mouse	1:800	Abcam[b]	AB5920
SP	Guinea pig	1:1000	Abcam[b]	AB1053
PGP 9.5	Mouse	1:200	ABD Serotec[c]	7863-1004
nNOS	Mouse	1:500	Santa Cruz Biotechonology[d]	SC-5302
HCN4	Mouse	1:200	StressMarq Biosciences[e]	SMC-320D
Secondary				
Goat^{Cy3}	Donkey	1:300	Chemicon[a]	AP180C
Mouse^{Cy3}	Donkey	1:300	Chemicon[a]	AP192C
Mouse$^{DyLight\ ®488}$	Donkey	1:300	Abcam[b]	AB96875
Mouse^{FITC}	Donkey	1:100	Jackson Immunoresearch[f]	715-095-151
Sheep^{FITC}	Donkey	1:100	Chemicon[a]	AP184F
Guinea pig^{FITC}	Donkey	1:100	Chemicon[a]	AP193F

[a] Chemicon International, Temecula, CA, USA.
[b] Abcam, Cambridge, UK.
[c] AbD Serotec, Kidlington, UK.
[d] Santa Cruz Biotechnology, Dallas, TX, USA.
[e] StressMarq Biosciences Inc., Cadboro Bay, Victoria, Canada.
[f] Jackson Immunoresearch, West Grove, PA, USA.

immunohistochemical method for choline acetyltransferase (ChAT) which is the crucial enzyme in acetylcholine synthesis (Nachmansohn et al., 1946). Recently, immunohistochemical labeling for ChAT has been approved as the most reliable cholinergic marker for parasympathetic nerve fibers (Arvidsson et al., 1997; Hoover et al., 2004; Saburkina et al., 2010; Pauza et al., 2014a,b). Therefore, this study was designed to simultaneously determine the distribution of SAN cells and their innervation in whole mount preparations of the rabbit heart employing immunohistochemical methods for reliable determination of autonomic innervation and SAN cells in order to compare their morphologic patterns with those in other mammalian hearts examined previously (Crick et al., 1994, 1996, 1999; Pauza et al., 2013, 2014a,b; Roberts, 1991a,b; Roberts et al., 1989; Yamamoto et al., 2006).

2. Material and methods

2.1. Study material

Twenty two young (4–6 week old) New Zealand rabbits of either gender weighing 420–940 g were used in accordance with local and state guidelines for the care and use of laboratory animals (Permission No. 0206). Nineteen hearts were used for whole mounts and three hearts for transverse section preparations.

2.2. Whole-mount preparations

Following animal euthanasia, hearts were dissected from the chest, cleaned with 0.01 M phosphate buffer saline (PBS) and the coronary vessels were retrogradely perfused with PBS. Afterwards, the atria were dissected from the ventricles and the interatrial septum. Then, the atria were flattened and pinned in a Petri dish with a silicone bottom. The flattened specimen was fixed for 40 min at 4 °C in 4% paraformaldehyde solution in 0.01 M phosphate buffer (pH = 7.4). In order to decrease background light for laser scanning microscopic examination, tissues were bleached using a dimethyl sulfoxide and hydrogen peroxide solution, and dehydrated as reported previously (Dickie et al., 2006). Subsequently, whole-mount preparations were rehydrated through a graded ethanol series (in each for 10 min), washed 3× 10 min in 0.01 M PBS containing 0.5% Triton X-100 (Serva, Heidelberg, Germany). Non-specific binding was blocked for 2 h in PBS containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Afterwards, the specimens were incubated with an appropriate combination of primary antisera for 48–72 h at 4 °C (Table 1). Afterwards, whole-mounts were washed 3 times for 10 min in 0.01 M PBS and incubated with an appropriate combination of secondary antisera for 4 h at room temperature (Table 1). Four combinations of antisera were used in this study in order to identify: (1) the SA nodal cells and cholinergic neural components (HCN4 + ChAT), (2) the biphenotypic neuronal somata and nerve fibers (ChAT + nNOS and ChAT + TH) and (3) general distribution of nerves, nerve fibers and neuronal somata as well as adrenergic neural structures and small intensively fluorescent (SIF) cells (PGP9.5 + TH).

During the last stage, specimens were washed 3 times for 10 min in 0.01 M PBS, mounted with Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA), cover slipped and sealed with clear nail polish.

2.3. Sectioned tissue preparations

In order to obtain transverse sections of walls of the rabbit RA and RRCV, three rabbit hearts were dissected, perfused and prefixed as described above. In order to compare the densities of NFs distributed between cardiac cells positive for HNC4 from distinct localities on RRCV, the SAN region was comparatively divided into three parts as described previously (Pauza et al., 2013, 2014a,b). SAN cells were categorized into three groups: SAN cells distributed on the medial part of the RRCV were considered to be the head of the SAN, the lateral ones that extended along the terminal groove to be the body, and their dorsal portion allocated toward the RRCV as the SAN tail (Fig. 1a). Following 40 min fixation at 4 °C in 4% paraformaldehyde solution in 0.01 M phosphate buffer (pH = 7.4), tissues were washed 3× 10 min in PBS and cryoprotected by immersion in PBS containing 20–25% sucrose until their complete sinking (4 °C, 24 h). Following cryoprotection, the tissues were frozen using a tissue-freezing medium (Triangle Biomedical Sciences, USA) for sectioning. The pieces of the sampled tissues were orientated on a cryomicrotome stage so that the slice cutting plane would be perpendicular to the interatrial septum, right atrial wall and the terminal groove and all layers (epicardium, myocardium and endocardium) could be well discernible in the slice. Tissues were sectioned into 20 μm slices using a cryomicrotome HM