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Original article

Innervation of sinoatrial nodal cardiomyocytes in mouse. A combined approach using immunofluorescent and electron microscopy ☆

Dainius H. Pauza [a,*], Kristina Rysevaite [a], Hermanas Inokaitis [a], Marius Jokubauskas [a], Audrys G. Pauza [a], Kieran E. Brack [b], Neringa Pauziene [a]

[a] Institute of Anatomy, Faculty of Medicine, Lithuanian University of Health Sciences, Kaunas, Lithuania
[b] Department of Cardiovascular Sciences and NIHR Leicester Cardiovascular Biomedical Research Unit, University of Leicester, Leicester, UK

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ABSTRACT

Fluorescent immunohistochemistry on the cardiac conduction system in whole mount mouse heart preparations demonstrates a particularly dense and complex network of nerve fibres and cardiomyocytes which are positive to the hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (HCN4-positive cardiomyocytes) in the sinoatrial node region and adjacent areas around the root of right cranial vein. The present study was designed to investigate the morphologic and histochemical pattern of nerve fibres and HCN4-positive cardiomyocytes using fluorescent techniques and/or electron microscopy. Adrenergic and cholinergic nerve fibres together with HCN4-positive cardiomyocytes were identified using primary antibodies for tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), and the HCN4 channel respectively. Amid HCN4-positive cardiomyocytes, fluorescence and electron microscopy data demonstrated a dense distribution of nerve fibres immunoreactive for ChAT and TH. In addition, novel electron microscopy data revealed that the mouse sinoatrial node contained exclusively unmyelinated nerve fibres, in which the majority of axons possess varicosities with clear mediatory vesicles that can be classified as cholinergic. Synapses occurred without any clear terminal connection with the effector cell, i.e. these synapes were of "en passant" type. In general, the morphologic pattern of innervation of mouse HCN4-positive cardiomyocytes identified using electron microscopy corresponds well to the dense network of nerve fibres demonstrated by fluorescent immunohistochemistry in mouse sinoatrial node and adjacent areas. The complex and extraordinarily dense innervation of HCN4-positive cardiomyocytes in mouse sinoatrial node underpins the importance of neural regulation for the cardiac conduction system. Based on the present observations, it is concluded that the occurrence of numerous nerve fibres nearby atrial cardiomyocytes serves as a novel reliable extracellular criterion for discrimination of SA nodal cardiomyocytes using electron microscopy.

1. Introduction

Previous studies have shown that atrioventricular conduction is preferentially sensitive to sympathetic activity, whereas sinoatrial (SA) automaticity is especially responsive to parasympathetic influence [1,2]. Despite this, the anatomical evidence suggests that cholinergic innervation is dense throughout the cardiac conduction system of many mammalian species, including human [3–9]. Contradictions between the physiological effects of autonomic stimulation and the neurochemical phenotype of nodal innervation were enhanced by our recent findings from whole mount mouse heart preparations [10,11]. In these studies, double labelling fluorescent immunohistochemistry demonstrated a dense, widespread and complex network of cholinergic and adrenergic nerve fibres in and around the SA and atrioventricular nodal areas. This was similar in neighbouring areas around the root of right cranial vein, below the orifice of the coronary sinus and along the atrioventricular ring. Whilst the morphology of HCN4-positive or SA nodal cardiomyocytes has been investigated previously [12–20], the reported network of nerve fibres was so unusual with respect to other cardiac regions, that these areas were considered worthy of further examination.

To do this, it is necessary to perform immunohistochemistry for HCN4-positive cardiomyocytes, cholinergic and adrenergic nerve fibres. However, fluorescent microscopy is spatially limited and does not allow for the structural relationships of distinct nerve fibres with cardiac myocytes to be visualized. Therefore, electron microscopy is necessary to facilitate discrimination of cells immunohistochemically labelled for distinct antigens and the correct identification of myocyte/neuronal morphology. Therefore, the present study was designed to examine the morphology and innervation of HCN4-positive or SA nodal cardiomyocytes using two distinct imaging methods—fluorescent immunohistochemistry

☆ Conflicts of interest: All authors have reported that they have nothing to disclose.

* Corresponding author at: Institute of Anatomy, Faculty of Medicine, Lithuanian University of Health Sciences, A. Mickeviciaus Street 9, Kaunas LT-44307, Lithuania. Tel.: +370 37 327313, +370 682 39366(Mobile); fax: +370 37 220733.
E-mail address: dainius.pauza@lsmuni.lt (D.H. Pauza).

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and transmission electron microscopy. The aims of this study are to determine (1) the distribution of HCN4-positive cardiomyocytes in relation to network of nerve fibres on the sinuses of right cranial and caudal veins, (2) the density of nerve fibres within the neural network of the SA nodal area, and (3) how autonomic nerve fibres are related to HCN4-positive cardiomyocytes in the mouse SA nodal area using electron microscope method.

2. Materials and methods

Thirty two 2–4 month-old C57BL/6J-linear mice of either sex were used for this study. Animals were deeply anesthetized with ether and euthanized by cervical dislocation in accordance with local and state guidelines for the care and use of laboratory animals (Permission No. 0206), which also conform to the European Union directive on the protection of animals for scientific research (2010/63/EU).

2.1. Whole mount fluorescent immunohistochemistry

Five mice were used for whole mount preparations to immunohistochemically identify intrinsic cardiac nerve fibres and HCN4-positive cardiomyocytes as previously described [10]. In brief, following euthanasia and a medial thoracotomy, a metal catheter was inserted transmyocardially into left ventricular cavity, where hearts were perfused in situ with phosphate buffered saline (PBS, 0.01 M) at room temperature via the coronary arteries by the aid of special gear that allowed perfusion using constant pressure (100–110 mm Hg). Once cardiac perfusion was confirmed and coronary blood was washed through, hearts were removed from the chest and placed into a dissecting dish containing cold 0.01 M PBS. The left and right atrial walls, appendages and interatrial septum were separated and pinned on a silicone pad in a dissecting dish, for fixation for 25 min at 4 °C in 4% paraformaldehyde (PFA) solution in 0.01 M phosphate buffer (PB, pH 7.4).

To decrease tissue autofluorescence, the flattened tissues were cleared using an ethanol with dimethylsulfoxide (20%, DMSO), hydrogen peroxide in ethanol (6%, H_2O_2) and dehydrated through a graded ethanol series as reported by Dickie et al. [21]. The whole-mount preparations were then rehydrated with 10-minute successive washes through a graded ethanol series, washed, and permeabilized in 3 × 10-minute changes of 0.01 M PBS containing 0.5% Triton X-100 (Carl Roth, Karlsruhe, Germany). Nonspecific binding was blocked for 2 h in PBS containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.5% Triton X-100. Preparations were subsequently washed (3 × 10 min) in 0.01 M PBS and incubated in a mixture of two primary polyclonal antibodies to identify sympathetic and cholinergic nerve fibres and SA nodal cardiomyocytes for 24–48 h in a dark humid chamber at 4 °C (Table 1). After three 10 min-washes in 0.01 M PBS, the whole-mount preparations were incubated in an appropriate combination of secondary antibodies for 4 h in a dark humid chamber on a shaker stage at room temperature (Table 1). All antibodies were diluted in 0.01 M PBS. Finally, the specimens were washed in 0.01 M PBS, incubated for 2 h in a DMSO and PBS mixture, 1:4 (*v:v*) ratio, and mounted in Vectashield Mounting Medium (Vector Laboratories, California, USA). A cover slip was placed on the tissue and then sealed with clear nail polish. Positive and negative controls [omission of primary and or secondary antibodies] were used.

The stained preparations were analysed utilizing an AxioImager Z1 fluorescence microscope equipped with filters to observe the isothiocyanate (FITC) and cyanine (Cy3) tagged secondary antibodies along with an Apotome2 and digital monochrome camera AxioCam MRm using AxioVision software (version 4.8.1., Zeiss, Gottingen, Germany). When necessary, the same whole-mount preparations were additionally analysed and photographed employing a laser-scanning microscope (LSM 700 with ZEN 2010 software, Zeiss, Jena, Germany).

Table 1
Primary and secondary antisera used in the study.

Antigen	Host	Dilution	Supplier	Catalogue number
Primary				
ChAT	Goat	1:100	Chemicon[a]	AB144P
TH	Rabbit	1:500	Chemicon[a]	AB152
TH	Sheep	1:800	Chemicon[a]	AB1542
PGP 9.5	Rabbit	1:500	AbD Serotec[b]	7869-0504
HCN4	Rabbit	1:100	Chemicon[a]	AB5808
Secondary				
$Goat^{Cy3}$	Donkey	1:300	Chemicon[a]	AP180C
$Rabbit^{Cy3}$	Donkey	1:300	Chemicon[a]	AP182C
$Rabbit^{FITC}$	Donkey	1:100	Chemicon[a]	AP182F
$Sheep^{FITC}$	Donkey	1:100	Chemicon[a]	AP184F
$Rabbit^{Biotin}$	Donkey	1:100	Chemicon[a]	AP182B

[a] Chemicon International, Temecula, California, USA.
[b] AbD Serotec, Kidlington, UK.

2.2. Heart sections fluorescent immunohistochemistry

In order to verify the presence of SA nodal cardiomyocytes in remote sites from typical regions, two hearts were fixed for 4 days at 4 °C in 4% PFA in 0.01 M PB (pH 7.4). Hearts were embedded in paraffin and sectioned into 4–5 μm thick slices employing a rotatory microtome (HS 355S, Microm, Walldorf, Germany) and applied onto Superfrost Ultra Plus microscope slides (Thermo Scientific, Braunschweig, Germany) and dried for up to 12 h at 50 °C. Sections were rehydrated and subjected to antigen retrieval in Tris–EDTA buffer (pH 9) at 90 °C for 20–40 min in a microwave oven (HistosPro, Milestone, Sorisole, Italy). Double labelling immunohistochemistry for HCN4 along with sympathetic and cholinergic neuronal markers was followed according to protocol described above and cover-slipped for analysis. A standard Masson's trichrome staining procedure was used to differentiate connective tissue, working myocardium and conduction system. In addition, a Periodic acid–Schiff reaction was used to detect higher level of glycogen in SA nodal cardiomyocytes.

2.3. Transmission electron microscopy (TEM)

For TEM examination, 13 mice of either sex were used. Animals were prepared according to the procedures used for whole mount tissue preparation described above. However, when the hearts were washed out from the blood, perfusion was switched to a solution containing 2% PFA and 0.25% glutaraldehyde in 0.1 M PB (pH 7.4). After fixation, hearts were excised from the chest and tissue samples of 1–2 × 2–3 mm from three defined sites (Fig. 1), were extirpated using a dissecting microscope, fine scissors and tweezers in a Petri dish containing 0.1 M PBS. Tissue samples were postfixed in the original fixative solution for at least 4 h at room temperature or overnight at 4 °C. Subsequently, tissue samples were treated with the electron microscopy fixative osmium tetroxide (1%) in 0.1 M PB (pH 7.4) for 2 h, dehydrated through a graded ethanol series and embedded in a mixture of resins Epon 812 and Araldite (Sigma-Aldrich, Steinheim, Germany) using an automated tissue processor (Lynx II, EMS, Hatfield, PA, USA). Before the final resin polymerization at 60 °C, tissue samples were carefully orientated for their transverse sectioning in flat embedding moulds under a stereoscopic microscope (Stemi 2000CS, Zeiss, Gottingen, Germany).

2.4. Electron microscopy immunohistochemistry

For EM immunohistochemistry, hearts of 12 mice were prepared, perfused and fixed as described above avoiding glutaraldehyde in the fixative, due to its strong inhibitory effect on tissue immunogenicity. Tissue samples were harvested from the same cardiac sites as for routine TEM (Fig. 1). The immunohistochemical reactions were performed on tissue samples in accordance with protocol suggested by Goehler