Short Communication

Measurement of Phosphodiesterase Activity in the Conduction System and Contractile Muscle of the Rat Heart: Evidence of Regional Difference

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ABSTRACT: Given that information regarding the biochemical regulation of cyclic adenosine monophosphate (cyclic AMP)-mediated signal transduction pathway in the atrioventricular node is limited, we quantitatively measured the phosphodiesterase activity in the conduction system and compared it with that in the contractile myocardium of rats by using the enzymatic fluorometric assay with the histochemical method (n = 4). The phosphodiesterase activities in the atrioventricular node, His bundle, and left ventricular myocardium were 87.9±7.8, 54.5±2.7, and 7.8±0.5 pmol/min/mg as cyclic AMP hydrolyzing speed, respectively (mean±SE). These regional differences imply that cyclic AMP-mediated signal transduction pathway in the conduction system may be more dynamically regulated than in the contractile myocardium.

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KEYWORDS: conduction system, phosphodiesterase activities, enzymatic fluorometric assay, histochemical analysis

Introduction

Although conduction system of the heart has been shown to possess different developmental, structural, and electrophysiological characteristics from contractile myocardium, information is still limited regarding the biochemical regulation of cyclic AMP-mediated signal transduction pathway in the conduction system.[1–3] Two methodological barriers have hampered efforts to begin to analyze the specialized electrical network. The first is a technical difficulty associated with obtaining samples from discrete portions of anatomically complex conduction system for further biochemical analysis.[1] The second is a lack of versatile yet sensitive assay, to quantify the phosphodiesterase activity in a small piece of tissue. In this study, we adopted a histochemical method using acetylcholinesterase stain to identify the conduction system and a highly sensitive non-radioactive enzymatic fluorometric

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assay to quantitatively measure phosphodiesterase activity in the atrioventricular node, His bundle, and left ventricular myocardium of rats.[2, 4–6]

Materials and Methods

Experiments were conducted in four male Sprague-Dawley rats of 7-9 weeks old weighing 250-280 g, which were obtained from Japan SLC, Inc. (Shizuoka, Japan). All experiments were approved by the Toho University Animal Care and User Committee (No. 16-51-322; 17-52-322) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Toho University.

Tissue preparation

The rats were anesthetized with thiopental sodium (40 mg/kg, i.p.). Immediately after euthanization, the hearts were removed and quickly plunged into liquid monochloro-difluoromethane pre-cooled with crushed dry ice (−80 ℃) as previously described.[2] Samples were stored at −80 ℃ until they were sectioned by cryostat as previously described.[2] It took <5 s to place the heart in liquid monochloro-difluoromethane after removing.

Each heart was mounted on the cryostat chuck oriented with the interventricular septum perpendicular to the plane of the chunk. The blade was positioned to section serially, beginning at the base of the heart, at an angle of 30°. In this way, the atrioventricular node and His bundle regions were cut in 20-μm-thick sections orthogonally to the endocardial surface and prepared as previously described.[2–4] Briefly, alternate sections mounted on gelatin-coated glass slides were stained with acetylthiocholine for acetylcholine esterase activity and with hematoxylin for counterstaining. The acetylcholinesterase stain, which stains conductive elements with an intense rust color, is the most commonly used method to identify specific regions of the conduction system.[2–4] The atrioventricular node was specifically identified by its anatomic location within the lower portion of the interatrial septum, the presence of the atrioventricular nodal artery, and its relationship with the fibrous trigone and the His bundle. The His bundle was localized by its position at the summit of the interventricular septum, being contiguous to, but anterior to the ventricles, separate from the atria, and anteromedial to the tricuspid valve. With acetylcholinesterase stain, proximal portions of the bundle branches were readily localized as they track from the His and common bundle regions.

The remaining frozen sections were stored under vacuum at −40 ℃ for at least 48 h to be dried. They were then brought to room temperature and microdissected under a stereomicroscope with the guide of stained sections. Care was taken during the microdissection process of each tissue of conduction system to avoid including surrounding fibrous tissue and the connective tissue matrix. Ventricular samples were obtained from contractile myocardium near the conductive elements. Sample weights were measured by using a fishpole balance (Amersil; Hillside, NJ, USA), which was constructed with a quartz fiber horizontally mounted by one end. Samples were dissected so that their weight ranged from 1 to 3 μg. A set of 6-tissue pieces was obtained from atrioventricular node, His bundle regions, and left ventricular myocardium in each heart, respectively. It takes <30 min to microdissect whole pieces of tissues from one heart under a stereomicroscope with the guide of stained sections, whereas the enzyme activities in freeze-dried tissues are known to be kept stable in the absence of water for more than 8 h at room temperature.[1–4]

Enzymatic assay of phosphodiesterase activity

Phosphodiesterase assay consisted of two parts, hydrolysis reaction of externally added cyclic AMP by phosphodiesterase in the tissue sample followed by measurement of residual cyclic AMP as previously reported.[6, 7] The former part was carried out under low magnification of a stereo dissecting microscope.[4] Samples from the conductive and contractile tissue were placed in an oil well rack consisting of a 20 × 120 mm block 5 mm thick drilled with 60 holes of 3 mm diameter.[4] A volume of 0.4 μL of SET buffer (0.25 mol/L sucrose; 0.1 mmol/L EDTA; 5.0 mmol/L Tris-acetate, pH 7.4) was placed on the bottom of the hole with a constriction pipette (Tanja Kyander-Teutsh; Ulm, Germany) to dissolve the sample. The holes were then filled with oil (40% hexadecane and 60% U.S.P. light mineral oil). Then, a volume of 0.4 μL of reaction mixture (100 mmol/L Tris-acetate, pH 7.4; 20 mmol/L KCL; 10 mmol/L $MgCl_2$; 0.4 mg/mL bovine serum albumin, 20 μmol/L cyclic AMP) was added to the samples from each region.[6] After incubation at 37 ℃ for 20 min, a volume of 1 μL of 0.1 mol/L NaOH was added to each well, which was heated at 80 ℃ for 30 min. A volume of 1 μL of aliquot was transferred from each oil well into a 10 × 75 mm Pyrex tube (Corning; New York, NY, USA), and the cyclic AMP concentration was assayed with enzymatic fluorometric method.[5, 6] The phosphodiesterase activities in the respective area of each heart were calculated by us-

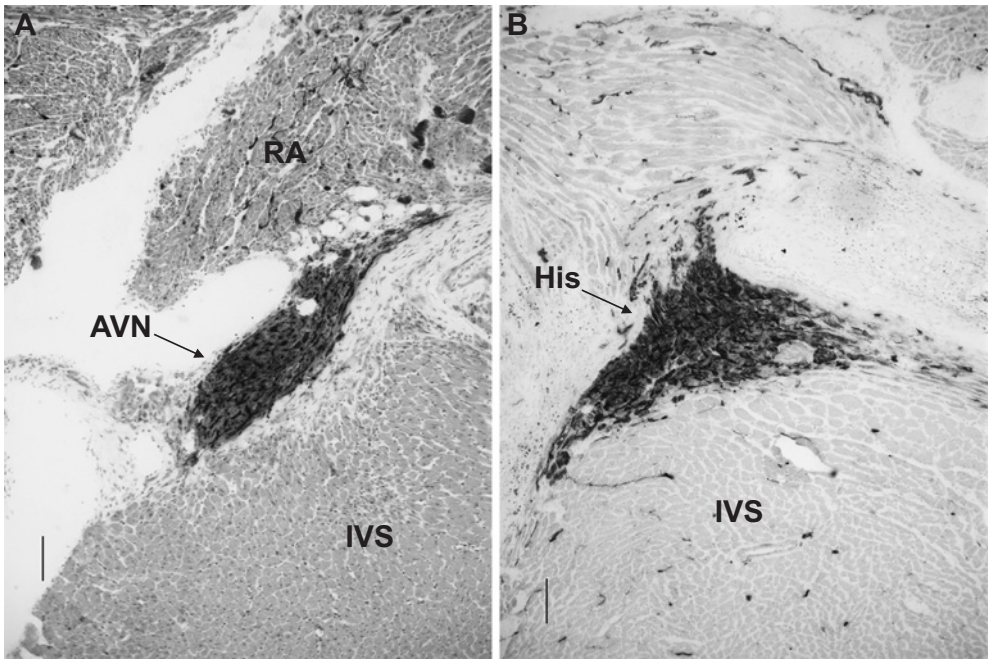

Fig. 1 Representative photomicrographs of atrioventricular node (AVN) and His bundle (His) areas stained for cholinesterase. The large dark areas are acetylcholinesterase-rich regions, indicating atrioventricular node (A) and His bundle (B). The vertical black line indicates a scale of 0.1 mm. RA: right atrium; IVS: interventricular septum.

ing the measured values of the 6-tissue pieces/region, which was then averaged to obtain mean phosphodiesterase activities of four hearts for each region.

Enzymes and substrates

All enzymes and chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) except for thiopental sodium (Ravonal® 0.5 g for Injection), which was purchased from Mitsubishi Tanabe Pharma Co. (Osaka, Japan).

Statistical analysis

Data are presented as the mean±SE. The statistical comparisons of mean values were evaluated by one-way repeated measures analysis of variance followed by Contrasts as a post hoc test. A p-value less than 0.05 was considered to be statistically significant.

Results

Identification of atrioventricular node and His bundle

Representative photomicrographs of acetylcholinesterase-rich portions of the atrioventricular node and His bundle areas are depicted in Fig. 1. The dark color of the atrioventricular node and His bundle were grossly distinguished from the light staining color of contractile tissues. As shown in Fig. 1A, the major axis of the sectional diameter of the atrioventricular node was 0.83 mm. The atrioventricular node and His bundle were able to be also identified in the unstained, freeze-dried sections with the naked eye, when the stained sections were used as a guide as previously described.[1)]

Phosphodiesterase activity

The phosphodiesterase activities in the atrioventricular node, His bundle, and left ventricle were summarized in Fig. 2, which were 87.9±7.8, 54.5±2.7, and 7.8±0.5 pmol/min/mg of dry weight as cyclic AMP hydrolyzing speed, respectively (n = 4). Significant differences were detected in each pair of the regions ($p < 0.05$).

Discussion

Given a lack of information regarding phosphodiesterase activity in the cardiac conduction system, we adopted histochemical method in addition to highly sensitive enzymatic assay technique, enabling to quantitatively measure phosphodiesterase activity in a small piece of tissue weighing 1-3 μg of dry weight. We have reported that the basal concentration of cyclic AMP in conduction system was similar to that in contractile myocardium,[2)] although the basal adenylylcyclase activity estimated as cyclic AMP production rate was approximately 2 times greater in the former than in the latter.[1)] In the present study, we

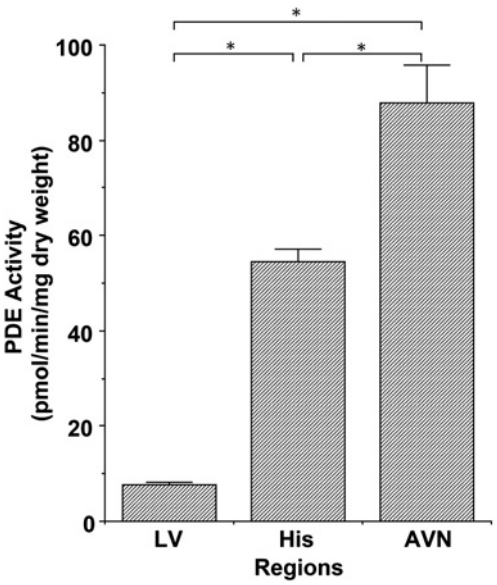

Fig. 2 Phosphodiesterase (PDE) activities in the left ventricle (LV), His bundle (His) and atrioventricular node (AVN) of rats. Data are expressed as mean ± SE ($n = 4$). *: Significant difference between the regions by $p < 0.05$.

showed that the basal phosphodiesterase activity assessed as cyclic AMP hydrolyzing speed was 11 and 7 times higher in the atrioventricular node and His bundle than in the left ventricle, respectively. These results may partly explain why the basal concentration of cyclic AMP in conduction system was similar to that in contractile myocardium, since the level of cyclic AMP depends on a balance between synthesis by adenylylcyclase and degradation by phosphodiesterase.

There are some limitations in this study. We could not confirm whether measured phosphodiesterase activity can be inhibited by phosphodiesterase inhibitor in discrete regions of the conduction system for each heart because of a limited amount of tissue sample. However, using the same assay method for phosphodiesterase activity as used in this study, we have demonstrated that typical phosphodiesterase inhibitor 3-isobutyl-1-methylxantine significantly inhibited the activity of phosphodiesterase from the bovine heart and rat gastrointestinal tissue in a concentration-related manner.[6, 7]

In conclusion, the phosphodiesterase activity was in the order of atrioventricular node >His bundle >>left ventricular myocardium. The regional differences of phosphodiesterase activity imply that cyclic AMP-mediated signal transduction pathway in the conduction system may be more dynamically regulated than in the contractile myocardium. Moreover, the difference of phosphodiesterase activities between atrioventricular node and His bundle might help to better understand the onset mechanisms of junctional tachycardia induced by β-adrenergic stimulation.

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Conflicts of interest: None declared.

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