RESEARCH ARTICLE

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Localization of Parvalbumin Calcium Binding Protein in the Rat Heart

Uraporn Vongvatcharanon[a,*] and Surapong Vongvatcharanon[b]

[a] Department of Anatomy, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.
[b] Department of Oral and Maxillofacial Surgery (Anesthesiology section), Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand.
* Corresponding author, E-mail: vuraporn@ratree.psu.ac.th

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ABSTRACT: Parvalbumin is a Ca^{2+} binding protein that functions as a relaxing factor in fast skeletal muscle fibers by acting as a delayed Ca^{2+} sink. As the cardiac contraction/relaxation cycle has to be constant and continuous, a precise rise and fall of intracellular calcium ions is required. Thus, cardiac muscles should contain certain Ca^{2+} binding proteins to buffer Ca^{2+} as in skeletal muscle fibers. Using immunohistochemistry techniques, we demonstrate that parvalbumin is found in atrial and ventricular cardiomyocytes, and is also located in the sinoatrial (SA) and atrioventricular (AV) nodes. The role of parvalbumin in cardiomyocytes may be related to the process of cardiomyocyte relaxation by facilitating Ca^{2+} transport from the myofibrils into the SR, similar to what occurs in skeletal muscles. In addition, parvalbumin may act to buffer Ca^{2+} concentrations in the SA and AV nodes.

KEYWORDS: parvalbumin, calcium binding protein, heart, SA node, AV node.

INTRODUCTION

The calcium ion (Ca^{2+}) plays an important role in contraction and relaxation of muscle fibers. In the fiber, the depolarization signal by the T tubule is transmitted to the sarcoplasmic reticulum (SR), resulting in the release of Ca^{2+} from the SR, that leads to an increased intracellular Ca^{2+} concentration in the myoplasm. Ca^{2+} binds to the contractile machinery (troponin C) resulting in fiber contraction. In the muscle relaxation phase, Ca^{2+} is removed by cytoplasmic proteins and transported back into the SR by SR Ca^{2+}-ATPase.[1] Parvalbumin, a high-affinity Ca^{2+} binding protein, has been proposed to be a relaxation factor in fast twitch muscle fibers, which acts as a Ca^{2+} sink that temporarily stores Ca^{2+} before uptake by the SR.[2] Therefore, the relaxation is dependent on the rate of Ca^{2+} binding to parvalbumin, and is independent of the energy.[3] It has been suggested that parvalbumin binds to Mg^{2+} at rest, thus Ca^{+2}, when released by the SR, would primarily bind to troponin C, because the Ca^{2+} binding to parvalbumin is postponed by the Mg^{2+} off rate.[4-5]

Parvalbumin was found at high concentration in fast - contracting skeletal muscle fibers of vertebrates[6,7] and in neurons.[8,9] However, it has been suggested that parvalbumin is not naturally expressed in the heart.[7,10] As the normal cardiac contraction/relaxation cycle is constant and continuous, a precise transient rise and fall of the number of intracellular calcium ions is required. Thus, cardiac muscle fiber should contain certain Ca^{2+} binding proteins that can facilitate Ca^{2+} translocation into the sarcoplasm in the same manner as in the skeletal muscles. Celio and Heizman,[7] who tried to localize parvalbumin in the heart by using parvalbumin antisera at a dilution of 1:20,000, concluded that there was no parvalbumin in the heart. However, their finding was not supported by other investigators. Therefore, by using various dilutions of parvalbumin antibody, this study investigates whether the heart (cardiomyocytes of the atrium and ventricle, including the sinoatrial and atrioventricular nodes) contains parvalbumin calcium binding proteins. The anti-parvalbumin monoclonal antibody used in this study is highly specific to parvalbumin calcium binding protein and does not react with other calcium binding proteins such as calmodulin, calbidin-D-28K, calretinin and troponin.[7] The specificity of the parvalbumin antisera was examined by many methods for example immunoelectrophoresis, immunoreplica technique and immunoprecipitation.[8] The result from this study may explain the pathology of heart failure caused by diastolic dysfunction and arrhythmia caused by Ca^{2+} overload, and may lead to new approaches to prevent heart failure or improve arrhythmia therapy.

Materials and Methods

Adult Wistar rats age 4 weeks were obtained from the Animals Unit, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. They were anesthetized by ether inhalation. The hearts and esophagus were removed and fixed in 10% formaldehyde. Following dehydration and embedding in paraffin, serial 5 μm sections were cut in both the horizontal and the coronal planes and mounted on TESPA coated slides. To localize SA nodes and AV nodes, the sections were dewaxed, rehydrated and stained with Masson Trichrome.

Localization of parvalbumin by using the immunohistochemistry method.

Heart sections alternating with those stained with Masson Trichrome and sections of esophagus were dewaxed, rehydrated and incubated sequentially with 0.3% Triton X-100 in Tris Buffered Saline (TBS) pH 7.2 (30 min), 3% H_2O_2 in methanol (30 min), 10% normal horse serum (Vector) in TBS (60 min), and finally with the monoclonal antibodies to mouse parvalbumin (Sigma) at dilutions of 1:2000, 1:5000, 1:10000, 1:15000 and 1:20000 in TBS for 48 hours at 4 °C. Then, the sections were rinsed with TBS and incubated with the biotinylated secondary anti-mouse antibody (anti-mouse IgG, Vector Laboratories, Burlingam UK), at a dilution of 1:200 in TBS for 2 hours at room temperature. After three rinses, the avidin-biotin-peroxidase complex was constructed using ABC reagent (Vector laboratories) and visualized using the chromogen-based visualization system, DAB (Vector laboratories). A positive control was generated by applying the same protocol to the esophageal section. A negative control was performed by omitting the primary antibodies. Finally, the sections were dehydrated in a graded series of alcohol, cleared in xylene and cover-slipped with DPX (Boehringer Ingelherm Bioproducts, Heidelberg, Germany). Images were captured with an Olympus DP-11 digital camera and image files were processed using Microimage software (Olympus).

Results

In the positive control, parvalbumin immuno-reactivity was observed in muscular layers of esophagus by using a 1: 2000 dilution of parvalbumin antibodies. It showed-up as a dark brown stain color, as demonstrated in Fig 1A. The negative control is shown in Fig 1B. The parvalbumin immunoreactivity was identified in the heart (atrial and ventricular cardiomyocytes, including the sinoatrial (SA) and atrioventricular (AV) nodes) by using the dilutions of 1: 2000 and 1: 5000 of parvalbumin antibodies. However, in the dilution 1:5000 of parvalbumin antibodies, parvalbumin immunoreactivity in the heart was slightly different from that of the negative control (not shown). Whereas, in a dilution of 1:2000 of parvalbumin antibodies, immunoreactivity in the interatrial septum and interventricular septum were very strong (Figs 2A, 3A), and different from that of the negative control (Figs 2B, 3B), suggesting that this dilution is optimal for localizing parvalbumin in the heart. Masson Trichrome staining showed the SA node at the wall of the proximal superior vena cava adjacent to right atrium (Fig 4A). The wall of the proximal superior vena cava also contains circularly disposed cardiomyocytes. The SA node is well demarcated from the vena caval musculature, as its cardiomyocytes packed with myofibrils were darkly stained. SA nodal cells are smaller in diameter than cardiomyocytes and myofibrils are sparse and concentrated near the periphery of the fiber leaving a clear central area (Figs 4B, 4C). The nuclei of the nodal fibers are round or oval, filling almost the entire diameter of the cell (Fig 4C). SA nodal cells show strong parvalbumin immunoreactivity using 1:2000 dilution of parvalbumin antibodies (Fig 4D). The cardiomyocytes in the wall of the proximal superior vena cava also exhibit parvalbumin immunoreactivity (Fig 4E) when compared to the negative control in Fig 4F. The coronal sections of the heart stained with Masson Trichrome showed an atrioventricular node (AV node) at the lower part of the interatrial septum astride the ventricular septum (Fig 5A). The AV node consists of thin modified muscle cells, which were stained more lightly than those of the atrial and ventricular cardiomyocytes, because of the few myofibrils present in their cytoplasm (Fig 5B). AV nodal cells showed strong parvalbumin immunoreactivity using parvalbumin antibodies at the dilution of 1:2000, and the immunoreactivity is stronger in the AV node than in the cardiomyocytes (Fig 5C).

Discussion

It is clear from our study that parvalbumin is found in atrial and ventricular cardiomyocytes, the SA node and the AV node. Interestingly, the AV node shows a stronger immunoreactivity for parvalbumin than do cardiomyocytes. The relative staining intensities presumably correspond to differences in the concentration of parvalbumin, suggesting that the concentration of parvalbumin in the AV nodes is higher than in cardiomyocyte. According to Celio and Heizman,[7] who localized the parvalbumin in fast contracting muscle fiber by using parvalbumin antibody at a dilution of 1:20,000, cardiac muscle

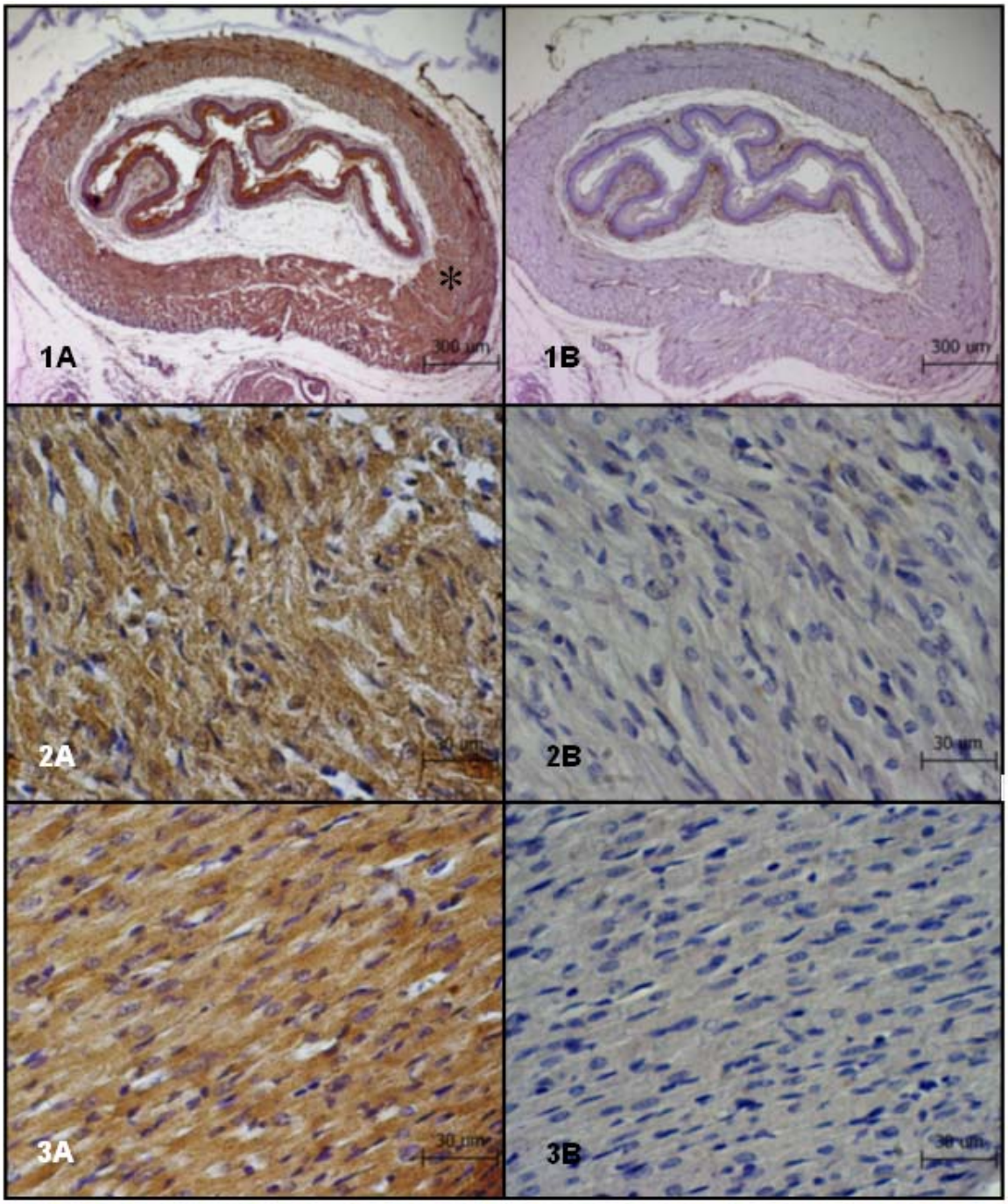

Fig 1. A: Cross section of the upper part of the esophagus stained with parvalbumin antibody at a dilution of 1:2000, the immuno reactivity is shown in the muscular layer of the esophagus (*). **B:** Negative control.

Fig 2. A: Coronal section of the interatrial septum of the heart, stained with parvalbumin antibody at a dilution of 1:2000, showing immunoreactivity (brown color) in the cytoplasm of all cardiomyocytes. **B:** Negative control.

Fig 3. A: Coronal section of the interventricular septum of the heart, stained with parvalbumin antibody at a dilution of 1:2000, showing immunoreactivity (brown color) in the cytoplasm of all cardiomyocytes. **B:** Negative control.

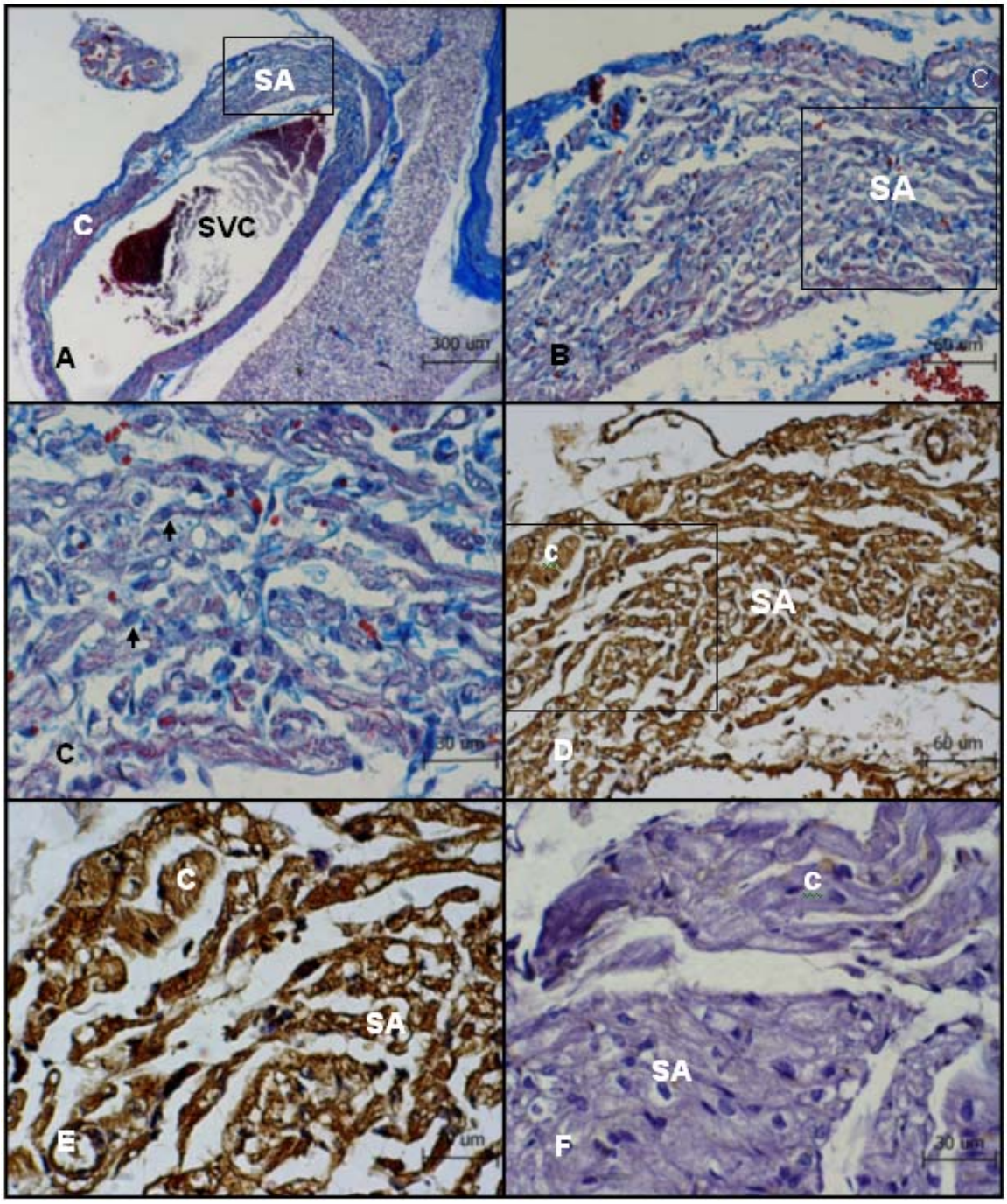

Fig 4. **A:** Cross section of the heart at the junction of the superior vena cava (SVC) and right atrium, stained with Masson Trichrome, showing the SA node (SA).
B: Higher magnification of the boxed area in **A** showing the SA node which is well demarcated from vena caval musculature.
C: Higher magnification of the boxed area in **B** showing the nuclei (arrow) of the nodal fibers; they are round or oval in shape, filling most of the area of the cells.
D: Cross section of the heart at the junction of SVC and right atrium, stained with parvalbumin antibody at a dilution of 1:2000, showing immunoreactivity (brown color) in the cytoplasm of nodal fibers (SA) and cardiomyocytes (C).
E: Higher magnification of the boxed area in **D**, stained with parvalbumin antibody at a dilution of 1:2000, showing the intensity of parvalbumin immunoreactivity in the SA node compared to cardiomyocytes (such as C).
F: Negative control section of the junction of the superior vena cava and right atrium showing no staining in the SA node and cardiomyocytes (C).

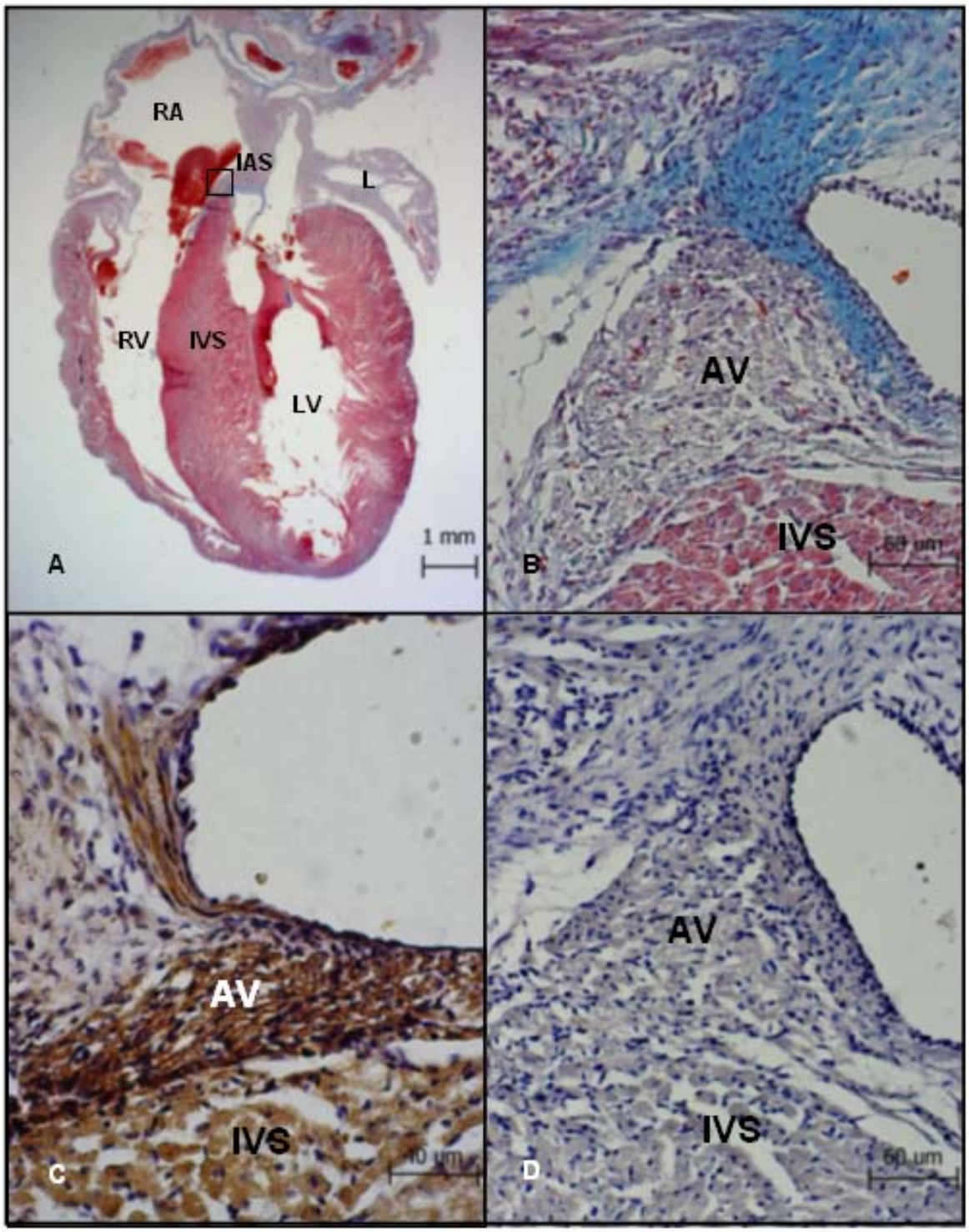

Fig 5. **A:** Coronal section of the heart, stained with Masson Trichrome, showing all chambers: right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV). Interatrial septum (IAS) and interventricular septum (IVS) are also shown.
B: Higher magnification of the boxed area in **A** showing the AV node which consists of thin modified muscle cells and is stained more lightly than those of the ventricular cardiomyocytes.
C: Coronal section of the heart, stained with parvalbumin antibody at a dilution of 1:2000, showing immunoreactivity (brown color) in the cytoplasm of nodal fibers and cardiomyocytes.
D: Negative control section showing no staining in the AV node and interventricular septum (IVS).

fibers were never labeled by parvalbumin antisera. However, in our study, we localized parvalbumin in the heart by using varied dilutions of parvalbumin antibodies, and found the optimal dilution to be at 1:2000. Interestingly, this dilution is the same as that used to localize parvalbumin in neurons of the brain,[11,12] suggesting that the concentration of parvalbumin in cardiac muscle is as high as in neurons but less than in fast-contracting and relaxing skeletal muscles. We believe that the role of parvalbumin in cardiac muscle fibers is to promote relaxation by shuttling Ca^{2+} from troponin C (TnC) to the sarcoplasmic reticulum, similar to its role in skeletal muscle.

An alteration of the parvalbumin levels may affect the contraction/relaxation cycle of muscle fiber as was seen in parvalbumin knockout mice, which have a prolonged contraction-relaxation cycle in their fast-twitch muscle.[13] It has been shown that deficiency in parvalbumin increases fatigue resistance in fast - twitch muscle in mice.[1] Therefore, in heart failure, where the heart relaxes very slowly after each contraction, we expect a decreased level of parvalbumin. Wahr and Szatkowski[3,14] indicated that parvalbumin is normally absent in cardiac muscle, so they transferred the parvalbumin gene to cardiomyocytes *in vivo* to enhance heart relaxation performance. We have shown here that parvalbumin does occur in atrial cardiomyocytes, ventricular cardiomyocytes, the SA node and with a high concentration in the AV node. It is possible that a patient with diastolic dysfunction in heart failure may have a deficiency of parvalbumin. If this can be proven, parvalbumin gene transfer may offer a new approach to treatment of diastolic dysfunction in heart failure therapy.

The role of parvalbumin in the SA and AV nodes is also interesting because in the AV node, the intensity of parvalbumin was higher than that in cardiomyocytes. The function of the SA node and the AV node is the generation and conduction of electrical impulses, which are different from those in cardiomyocytes, whose primary function is contraction. Therefore, we suggest that the function of parvalbumin in the SA and AV nodes may be similar to that in neurons, where parvalbumin acts as an intracellular Ca^{2+} - buffering transport protein[15-18]. It has been suggested that parvalbumin may influence the electrical properties and enzymatic machinery which modulate neuronal excitability and activity[9]. By binding Ca^{2+}, parvalbumin may prevent the activation of the Ca^{2+} activated K^+ current, resulting in reducing the after-impulse hyperpolarization and accommodation of the neuron. The shortening of the refractory period may allow the neuron to fire in rapid repetition and to recover more quickly from a presynaptic excitatory bombardment.[9] We suggest that parvalbumin in the SA and AV nodes may be involved in the generation and conduction of the electrical impulse by maintaining constant level of the intracellular calcium ion. It has been suggested that the neuron containing parvalbumin would be more resistant to cellular degeneration because of its greater capacity to buffer Ca^{2+}, thus protecting them from Ca^{2+} overload.[19] In arrhythmia caused by Ca^{2+} overload, a decreased level of parvalbumin may be considered as a possible cause, thus parvalbumin gene transfer may be helpful in treating this symptom.

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