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Morphometrical and Cytochemical Studies on the Isolated Single Cells from Adult Rabbit Heart†

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= Abstract = Isolation of intact single cardiac cells was carried out to investigate the shape, size and structure together with the cytochemical reaction in adult rabbit. Perfusion of the heart with calcium free Tyrode solution containing 0.04% collagenase resulted in 50% viability of single cardiac cells which were confirmed by trypan blue exclusion test and Janus green B stain. Isolated single cardiac cells from the left ventricle and atrium were both rod shape. The average length and width of the cells from the ventricle and the atrium were 132.9 μm and 10.6 μm, 132.0 μm and 12.3 μm respectively. The isolated single cells from the SA and the AV nodes were both oval shape and their long axis and short axis were measured as 33.8 μm and 25.0 μm, and 33.8 μm and 24.6 μm respectively. All the single cardiac cells contained abundant positive granules after McManus' periodic acid Schiff reaction. The single cells from the SA node and the AV node revealed fibrillar structures in their cytoplasm and the single cells from left ventricle and left atrium showed blue cross striations after Mallory's phosphotungstic acid hematoxylin stain.

Key Words: ***Single cardiac cell, Collagenase, Morphometry, Cytochemistry, Adult rabbit heart***

INTRODUCTION

There have been many studies on the heart in morphological and functional aspects. Some (Berry *et al.* 1970; Moller & Jensen 1972; James 1977) treated the heart as a whole *in vivo* or the removed heart *in vitro,* while others (Noma & Irisawa 1976) made small preparations of cardiac myocytes and analysed quite variable characteristics by the sites of the heart. But in these multicellular preparations, there always have been many limitations in the experimental method and interpretation of the results, such as the quasi-syncytial nature of the myocytes and accumulation or depletion of the ions in the extracellular space (Baumgarten and Isenberg 1977; Noble 1976). So there has been a need for the isolated single cardiac myocytes in studies of the biology, function, and structure of the heart. Since the pioneering work of the Vahouny *et al.* (1970) for isolating single cardiac cells from the adult rabbit heart, there have been many attempts to separate the heart into the isolated single cells mainly using enzymatic digestion method. Several methods have been reported for the isolation of intact, viable cardiac cells including mechanical disruption, tissue incubation with proteolytic enzymes (Vahouny *et al.* 1970) and organ perfusion with calcium-free media containing trypsin or collagenase with or without hyaluronidase (Berry *et al.* 1970). Many laboratories succeeded in obtaining single cardiac cells and reported the isolated cells had quite normal structures and metabolic properties similar to those observed in multicellular preparations, but most of their observations were made in a non-physiological solution specially made for the isolation procedure. And they reported ventricular myocytes beated spontaneously and most of them hypercontracted, rounded up and finally disaggregated when calcium concentra-

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tion was returned to the normal level of physiological solution (e.g. Vahouny *et al.* 1970; Berry *et al.* 1970) (calcium paradox; Zimmerman and Huelsmann 1966; Grinwald and Nayler 1980). Also there are not so many studies on the isolated cells of sinoatrial and atrioventricular node which are important structure for normal pacemaking of the heart. Recently a new method has been developed using collagenase (Taniguchi *et al.* 1981) and preincubation in a specially prepared solution (Isenberg and Kloeckner 1982) to aid in overcoming the calcium paradox. We attempted to get single cardiac cells from the adult rabbit heart using coronary perfusion method with calcium-free solution containing collagenase and to examine the size and shape as well as the cytochemical reactions on the isolated cells.

MATERIALS AND METHODS

Rabbits, weighing 1.0-1.5 kg, were anesthetized with pentobarbital sodium solution (30 mg/kg body weight) containing heparin (500 IU/kg body weight). The chest was opened under artificial respiration and the aorta was cannulated *in situ* to perfuse the coronary artery of the heart (Langendorff perfusion) with 80 ml of normal Tyrode solution containing in mM; NaCl 136.9, $NaHCO_3$ 6.0, KCL 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.33, HEPES 5.0, and pH was adjusted to 7.4 by adding NaOH. The heart was then dissected out and hung in the air while continuing the perfusion at a hydrostatic pressure of about 70 cm. After the blood was washed out, the perfusate was changed to calcium-free solution of about 80 ml. After the heart stopped beating and became relaxed completely, it was moved to another column filled with 50 ml of calcium-free solution containing crude collagenase (Sigma, type I) (0.4 mg/ml) and perfused for 60 minutes recirculating the enzyme solution in a little beaker with peristaltic pump. Following the enzymatic digestion of the connective tissue, the heart was again moved to the original column and perfused with 70 ml of high K-low Cl solution (storage solution) containing in mM; taurine 10, oxalic acid 10, glutamic acid 70, KCl 25, KH_2PO_4 10, HEPES 10, glucose 11, EGTA 0.5 and pH was adjusted to 7.4 by adding KOH. Then the heart was detached from the column and stored in the storage solution at 4°C. All the perfusate was saturated with 100% O_2 and kept at 36-37°C during the Langendorff perfusion.

After bathing in the storage solution for about 60 minutes at least, the heart was transferred under the dissecting microscope and cut into the right ventricle along the interventricular septum and through the atrioventricular groove into the right atrium. Opening the right atrium, the sinoatrial and atrioventricular nodes were identified (Moller and Jensen 1972) and cut into 3 or 4 pieces. Small pieces of the auricle of left atrium and the apical portion of the left ventricle were also resected. Then each piece of tissue was transferred to the Perspex chamber and teased apart mechanically by micropincettes and suspensions of isolated single cells were made for morphometry. The chamber was mounted on the stage of phase contrast inverted microscope and all the morphometrical and cytochemical studies were performed.

A small portion of the cell suspension was pipetted onto a specially designed chamber. To check the cell viability, staining with 0.33% trypan blue and supravital staining with Janus green B were performed. Then each length and width of the isolated single cardiac cells were measured by using micrometer in the eyepiece under the inverted microscope.

After completing the measurement, each isolated single cardiac cell was taken out with a pipette onto a microscopic slide glass. The transferred cell in the slide was confirmed to keep beating under the phase contrast inverted microscope. Zenker's fluid was added drop by drop, and the position of the cells was checked using X-Y axis parameter of the stage. After fixation, each slide was stained with McManus' periodic acid Schiff method and Mallory's phosphotungstic acid hematoxylin method. The slide was dehydrated, mounted and examined under the light microscope.

RESULTS

During the perfusion of the isolated heart with the normal Tyrode solution, spontaneous contractions continued. Single cells and cell clusters were obtained by treating with 0.04% collagenase in the calcium free Tyrode solution. Only the single cells were examined for further observation. About 50% of cells had viability examined through exclusion test in which living cells excluded trypan blue (TB) and through the staining with Janus green B (JGB) (Table 1).

Single, viable pacemaker cells were isolated from the sinoatrial (SA) and the atrioventricular (AV) nodes. Although these pacemaker cells were rod-shaped in the storage solution, they became found

Table 1. The cell viability evaluated by trypan blue (TB) exclusion test and Janus green B(JGB) stain

Stain	Sample No.	Viability(%)
TB	165	54.1
JGB	150	49.7

in an hour in normal Tyrode soluton (Fig. 3a, 5a). These cells contracted rhythmically in normal Tyrode solution and could be arrested by adding 10^{-7}M acetylcholine chloride (Ach) to the chamber. This fact shows that these pacemaker cells are nodal cells. These cells were observed under the phase contrast inverted microscope to have a relatively smooth and shiny surface (Fig. 3a, 5a). Also these cells had a pale cytoplasm and were smaller in size compared with the myocytes (Fig. 2, 3, 4, 5).

Both the SA node and the AV node cells continued beating in normal Tyrode solution at room temperature and the beat rate was increased almost linearly with the rise of temperature (Fig. 1). At 38°C, the beat rate of SA nodal cells was 229 per minute and that of AV nodal cells was 176 per minute.

Single myocytes isolated from the left atrium and the left ventricle showed rod-like appearances in storage solution. Exchange of storage solution with normal Tyrode solution showed no changes in morphology of single atrial and single ventricular myocytes (Fig. 5b). There were no differences in size and shape between atrial and ventricular

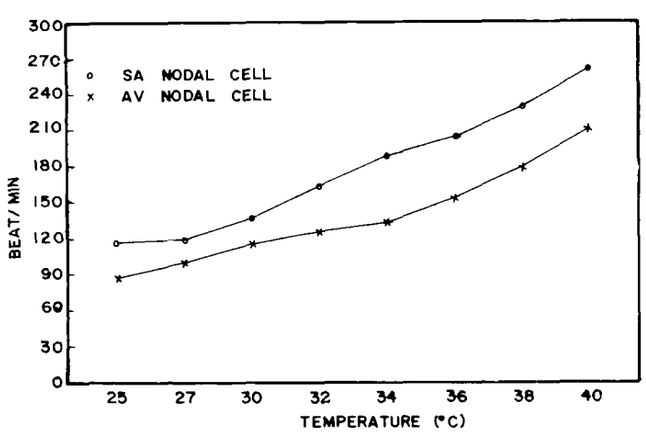

Fig. 1. Response of SA and AV node to the change of temperature. The beat rate increased almost linearly with the rise of temperature and that of SA node was generally higher than that of AV node by about 40 beat/min. At 38°C, SA node cells beat at 229/min, AV node cells beat 179/min.

myocytes and they did not beat in normal Tyrode solution. These myocytes were larger than nodal cells in length and revealed distinct striations and clear surfaces (Fig. 5b).

Morphometry of single cells: The isolated nodal cells were examined under phase contrast inverted microscope and measured their length (a) and width (b). The average size of the SA nodal cells was a=33.8±6.8 μm and b=25.0±4.8 μm, whereas that of the AV nodal cells was a=32.8±5.9 μm, b=24.6±5.1 μm. Between the SA and AV nodal cells, there was no significant difference in their size by T-test ($P<0.05$). The length (X) and width (Y) of myocytes were examined by the same method. The average size of the left atrial myocytes was X=132.0±32.2 μm and Y=12.3±3.8 μm, whereas that of the left ventricular myocytes was X=132.9±27.2 μm and Y=10.6±3.4 μm. There was no significant difference among these myocytes ($P<0.05$), however, significant difference in size between nodal cells and myocytes was recognized (Table 2).

Table 2. The size of isolated single cells from four portions of the rabbit heart (Mean and S.D.)

Tissue	Sample No.	Length(μm)	Width(μm)
SA	45	33.8± 6.8	25.0±4.8
AV	49	32.8± 5.9	24.6±5.1
LA	266	132.0±32.2	12.3±3.8
LV	500	132.9±27.2	10.6±3.4

AV, Atrioventricular node; LA, Left atrium
LV, Left ventricle; SA, Sinoatrial node

Cytochemistry of single cells: As a result of McManus' periodic acid Schiff reaction most of the single cells possessed PAS-positive granules throughout the cytoplasm and showed good preservation of fibrillar structures (Fig. 2, 4, 6). With the employment of Mallory's phosphotungstic acid hematoxylin stain, the atrial and ventricular myocytes showed distinct cross striation and well demarcated margin without haderent connective tissue fibers (Fig. 7). The nodal cells had fibrillar structures and did not show cross striations (Fig. 3 and 5).

DISCUSSION

Since the pioneering work done by Vahouny *et al.* (1970) of isolating the cardiac cell from adult rat

heart, the isolation procedure was improved to a large extent, but most of their cells showed calcium intolerance and also reports are rare for the pacemaking cardiac cells. Concerning the viability of isolated cardiac cell, Vahouny *et al.* 1970) reported 40% viability with trypsin and collagenase mixture in modified saline A solutions in rats. Rajs *et al.* (1978) obtained 40-50% viable cells with collagenase in Ficoll medium in adult rats, whereas Glick *et al.* (1974) showed 95% viability with collagenase in Ficoll medium in rats. The higher viabilities appeared in their reports are likely to be due to the difference of medium and enzymes. They observed in non- physiological solution and many of their cells hypercontracted and rounded up and finally disaggregated with the introduction of the normal Tyrode solution (Berry *et al.* 1970; Bishop and Drummond 1979).

In the present study, 50% of viable cells were obtained in normal Tyrode solution and this result was quite comparable to that of Isenberg and Klockner (1982). We could get sufficient amount of isolated single cardiac cells (nodal cells and myocytes) suitable for further studies on the morphometry and cytochemistry.

In the shape of isolated myocytes (ventricular and atrial cells) several researchers reported that the cells were rod-shape in rats (Vahouny *et al.* 1970; Glick *et al.* 1974; Rajs *et al.* 1978; Bishop and Drummond 1979), in guinea pigs (Bustamante *et al.* 1981; Bustamante *et al.* 1982), and in dogs (Vahouny *et al.* 1978). In the present study, myocytes of rabbit were also rod-shape and did not change their shape even in the exchange storage solution to normal Tyrode solution. No difference between interspecies was recognized.

James (1977) reported the nodal cells (SA and AV) as having slender or elongated form in the human heart and Taylor *et al.* (1978) reported that they observed pleomorphic forms of isolated nodal cells in rabbits. Taniguchi *et al.* (1981)'s description on the isolated nodal cells in rabbit were not concurrent to our study. He reported that the nodal cells were rod shape while we observed the cell as oval shape. This may be due to the difference of concentration of sodium bicarbonate. We used 6 mM of Sodium bicarbonate. In the present isolation procedure, the nodal cells were rod-shape in calcium-free solution in which the nodal cells were not beating and they became oval in normal Tyrode solution in which the cells beated regularly and spontaneously. This supports that the shape of nodal cells may change with the isolation medium.

All the myocytes beating in normal Tyrode solution were suggested to be dying cells and the beats were described to be the phenomenon of calcium paradox (Isenberg & Klockner 1982). In normal Tyrode solution, the nodal cells kept contraction rhythmically and intact myocytes did not beat. So the pacemaker cells are the oval-shaped nodal cells and this was supported by the changing pattern of beat dependent on the temperature.

On the size of isolated single myocytes, Vahouny et al. (1970) reported that the length and width were 50-100 μm and 10-50 μm respectively in rats. Other investigators reported the similar size in different animals (Glick *et al.* 1974; Vahouny *et al.* 1978; Bustamante *et al.* 1981). In our study, isolated myocytes in rabbit were similar to in width, but slightly larger than that of other animals in length. Isolated nodal cells in our experiment were also larger and were oval-form compared to round form of other reports (Taniguchi *et al.* 1981; Nakayama *et al.* 1984). Depending on our results of cytochemical staining isolated cell showed well preserved internal structure.

The isolation method have been established in our laboratory and it could be implemented by such as cardiac electrophysiology and to clinical cardiology.

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LEGENDS FOR FIGURES

Fig. 2. Isolated single cardiac cell from SA node. Note the abundance of red PAS positive materials in the cytoplasm. PAS stain, X400.

Fig. 3. Isolated single cardiac cell from SA node. A small amount of blue fibrillar structure is seen. PTAH stain, X400.

Fig. 3a. Isolated single cardiac cell from SA node (arrow). Note the smooth surface under the phase contrast inverted microscope. X200.

Fig. 4. Isolated single cardiac cell from AV node. Findings are similar to SA node. PAS stain, X400.

Fig. 5. Isolated single cardiac cell from AV node. findings are similar to SA node. PTAH stain, X400.

Fig. 5a. Isolated single cardiac cell from AV node under the pahase contrast inverted microscope. X400.

Fig. 5b. Isolated single cardiac cell from left ventricle. Note the well preserved cross striations and cell margin under the phase contrast inverted microscope. X200.

Fig. 6. Isolated single cardiac cell from left ventricle. Many granular PAS positive materials are well preserved. PAS stain, X400.

Fig. 7. Isolated single cardiac cell from left ventricle. Note well preserved blue striation. PTAH stain, X400.

*The bar in the figures (2-7) indicates 15 μm.

= 국문초록 =

분리된 토끼 단일 심근세포의 형태 계측학적 및 세포화학적 연구

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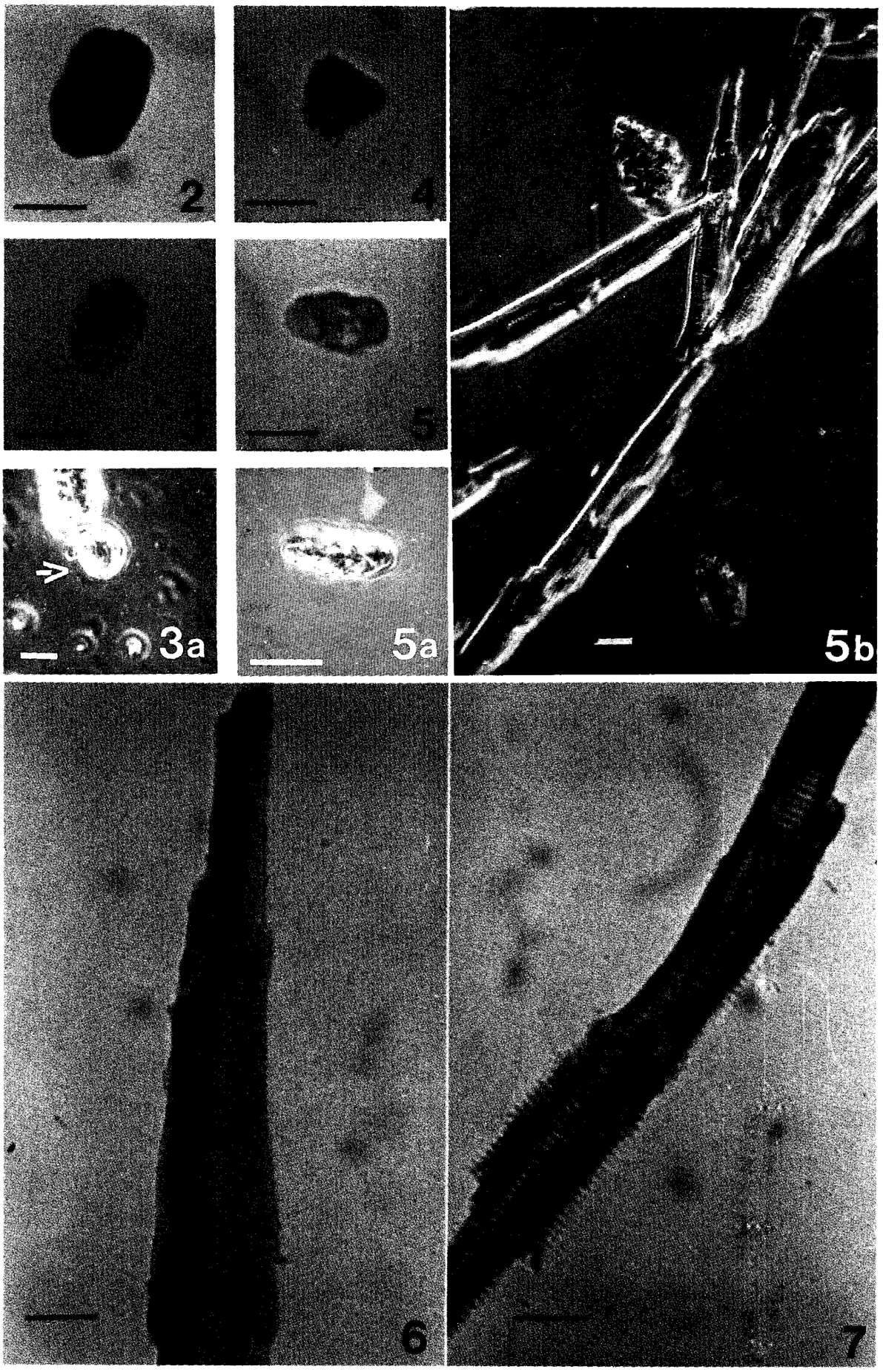
백상호 · 황덕호* · 박영배**

단일심근 세포의 형태와 크기, 그리고 세포화학적 특성을 밝히기 위하여 단일심근세포의 분리실험을 행하였다. 0.04% collagenase가 함유된 Ca-free Tyrode 용액으로 심장관류를 시행하여 분리된 단일 심장세포 부유액을 얻었다. 이들 단일세포 중 결절심근 세포는 자발적으로 수축을 계속하고 있음이 확인되었고 분리된 모든 심근세포 50%가 생존되어 있음을 trypan blue 생체 염색과 Janus green B 초생체 염색으로 확인하였다. 좌심실과 좌심방의 심근세포는 막대 형태로서 각각 크기가 132.9×24.6 um이었다. 모든 단일심근세포에서 PAS 염색에 양성으로 반응되는 입자가 풍부하게 보였다. 동방결절과 방실결절의 단일심근세포의 세포질 내에는 fibrillar한 구조물이 출현되었으며 PTAH 염색으로 푸른 횡문이 보였다.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