A Simple Method for Isolation of Cardiomyocytes from Adult Rat Heart

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A simple, economic, and sparing method for isolation of cardiomyocytes from adult rat heart is proposed. Ultrastructure of cardiomyocytes from suspension of freshly isolated cells was studied by light and transmission electron microscopy. The isolated cardiomyocytes were viable, had characteristic shape and size, and retained their normal structure.

Key Words: *cardiomyocytes; collagenase; pronase; heart perfusion; enzymatic treatment of the heart*

Cardiomyocytes were for the first time isolated from fetal heart in 1952 [6]. Methods for their isolation by mechanical separation or enzymatic treatment of the whole heart or its fragments were developed in recent decades. The results of 30-year development of methods for cell isolation from cardiac tissue are amply discussed [2]. Cardiomyocytes are isolated from mouse, rat, cat, dog, rabbit, cattle, and human hearts. Many modifications of enzymatic method are known [4,7].

The procedure of cardiomyocyte isolation includes a series of obligatory stages: heart washing from blood, treatment with calcium-free medium, perfusion of the whole heart or incubation of its fragments with enzyme solution containing collagenase, pronase, hyaluronidase, trypsin (each of the enzymes alone or in various combinations), resuspending of the heart tissue and separation into individual cells, washing of cells from enzyme solution. All these steps make the known methods of cardiomyocyte isolation difficult [1,2,4,7,9,10]. Moreover, they are not devoid of some shortcomings impeding operations with fresh-isolated cardiomyocytes. These shortcomings are the use of high concentrations of enzymes and/or prolonged treatment of the heart with these enzymes, which is fraught with the possibility of damage to the plasma membranes and membrane-bound receptors; solutions for washing, separation, and storage of cardiomyocytes contain, in addition to the standard salts, other components (amino acids, antibiotics, vitamins, ATP, EGTA, BSA), which is justified when special problems are solved, but precludes unambiguous interpretation of the results; numerous stages of the isolation process, when, in addition to perfusion and resuspending the cells are passed through filters, shaken for a long time, repeatedly centrifuged, are fraught with aggravation of mechanical injuries to the cells.

The aim of this study was to develop a simple method for isolation of adult rat cardiomyocytes, based on the use of simple saline and lower concentrations of enzymes.

MATERIALS AND METHODS

The most attractive methods, as regards the enzyme concentrations and duration of enzymatic treatment of the myocardium, are described previously [3,5,8]. Our study was carried out on Wistar rats (250-400 g). Sigma reagents were used for isolation. All solutions were prepared on deionized water. After decapitation the hearts were immediately removed and placed into ice-cold isolation medium. Standard saline or nutrient medium (medium 199, Krebs—Henseleit buffer, Thirode, Krebs—Ringer solutions, 0.15 M NaCl, *etc.*) can be used at this stage. The heart was placed into perfusion chamber as rapidly as possible and cannulated

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through the aorta. The heart was washed from blood with Krebs—Henseleit buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.25 KH_2PO_4, 1.3 $MgSO_4$, 10 glucose, 1.2 $CaCl_2$, and 10 HEPES (pH 7.4) for 5-10 min at 35°C. During the perfusion the buffer was saturated with a mixture of oxygen and carbon dioxide (carbogen) or with pure oxygen. The buffer was pumped through the heart by means of peristaltic pump at a rate of 4 ml/min. After the heart was washed from blood, it was perfused with calcium-free Krebs—Henseleit buffer for 5 min. It is noteworthy that many reagents can contain appreciable admixture of calcium, which impedes separation of cardiomyocytes and reduces the number of isolated cells. However, we do not recommend adding EGTA or EDTA into solution, because this is a special damaging factor and can lead to enzyme inhibition. Then the heart was perfused with Krebs—Henseleit buffer with low Ca content (<0.25 mM), pH 7.4, with collagenase (0.2 mg/ml) and pronase (0.1 mg/ml) for 20 min. During the next 20 min the heart was treated with buffer with pronase alone (0.1 mg/ml), pH 7.4. The duration of enzyme treatment can be reduced to 10-15 min at each of the stages, but then the cell yield will be much lower. The younger is the animal, the less time is needed for heart processing. The enzyme solution for heart treatment is used repeatedly, this reducing the amount of enzymes needed for one isolation. The next step was to wash the heart from enzymes, for which it was perfused with low-Ca buffer (pH 7.4) for 10 min at 35°C. Washed heart was removed from the cannula and placed into 10 ml Krebs—Henseleit buffer with Ca concentration of 0.25-1.00 mM After removal of large vessels, the heart was cut into 1-2-mm^2 fragments and carefully resuspended for 1 min with a large automated pipette (for better convenience, the tip of the headpiece was obliquely cut; the edges should be very smooth). In case of incomplete separation, the suspension was filtered through two gauze layers in order to remove the remaining fragments. Pushing (grinding) through Nylon filter is not recommended, because this increases the number of damaged cells. After this procedure the cells are ready for use and storage. The cells were stored in Krebs—Henseleit buffer with 1 mM Ca concentration (pH 7.4) at ambient temperature. If other cells and cardiomyocyte fragments are to be separated, the cells can be precipitated (50*g*, 3-5 min) and suspended in a fresh solution to the needed concentration. In order to prolong the life span of isolated cells, cardiomyocyte suspension should be stored at 4-8°C.

Viable cells were counted in a hemocytometer using 0.4% Trypan blue for visualization of damaged cells. The cells were examined under a Biolan-1 optic microscope and photographed with a Hitachi digital camera. For electron microscopy, freshly isolated cells were precipitated at 50*g* for 5 min and the precipitate was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4°C. After fixation the cells were washed twice in cacodylate buffer, fixed in 1% osmium tetroxide, and washed twice in cacodylate buffer. The resultant material was dehydrated in ascending alcohols and embedded in araldite. Ultrathin sections (30-60 nm) were sliced on an Ultrotom 111, mounted onto grds with Formvar coating, and contrasted by 2% uranylacetate and lead citrate. The preparations were examined under a JEM-100CX11 electron microscope with aperture diaphragm 25-30 μ at ascending voltage of 80 kV.

RESULTS

By this method we obtained 10-12×10^6 isolated cardiomyocytes; 70-80% cells were viable. The number of viable cells (in %) virtually did not change for 4 h and decreased to 50% during the next 2 h. The cells had a classical cylindrical shape with well-discernible transverse striation (Fig. 1, *a*, *b*) and uneven terminals at the intercalated discs (Fig. 1, *a*). Cell size varied from 10 to 25 μM (width) and from 80 to 130 μM

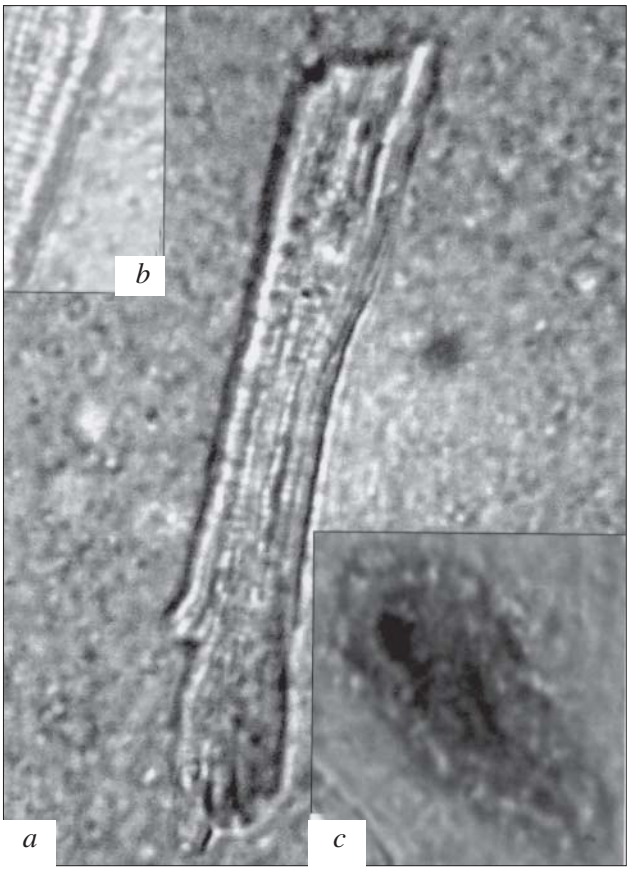

Fig. 1. Isolated adult rat cardiomyocyte in storage medium with 0.4% Trypan blue. *a*) normal cardiomyocyte, ×1000; *b*) fragment of normal cardiomyocyte, ×1500; *c*) dead cardiomyocyte filled with dye, ×1000.

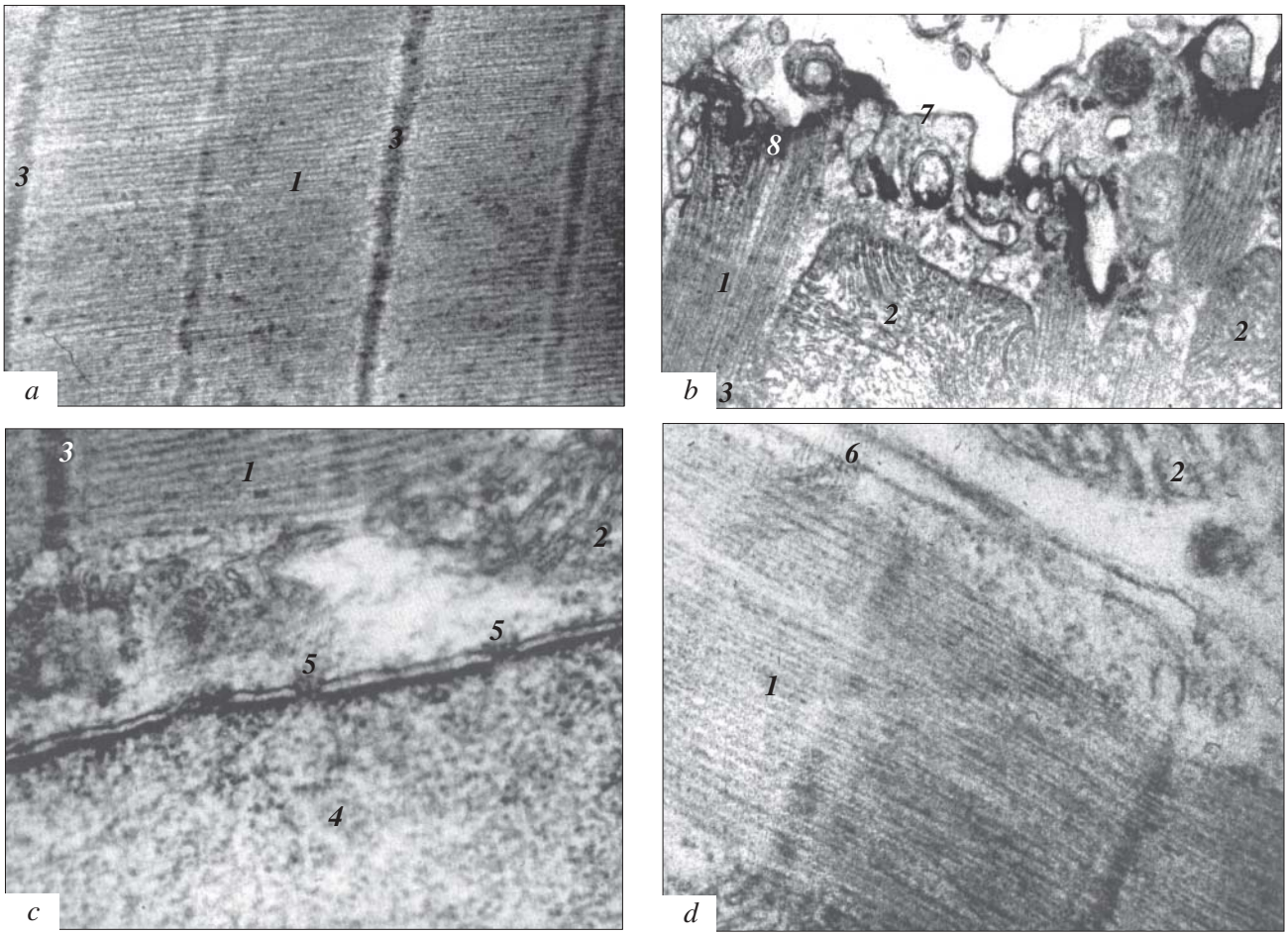

Fig. 2. Longitudinal section of isolated cardiomyocyte. *a*) myofibril fragment, ×32,500; *b*) intercalated disk area, ×26,500; *c*) fragment of nucleus with nuclear pores, ×53,300; *d*) myofibril fragment with adjacent sarcoplasmic reticulum and mitochondria, ×53,800. *1*) myofibrils; *2*) mitochondria; *3*) Z band; *4*) nucleus; *5*) pores in nuclear membrane; *6*) sarcoplasmic reticulum; *7*) sarcolemma; *8*) site of myofibril fixation.

(length). Intact cardiomyocytes in medium with 0.4% Trypan blue remained unstained half-transparent (this indicating the intactness of its membrane) against the stained background (Fig. 1, *a*). Cardiomyocytes damaged during isolation were shorter and wider, with rounded ends, stained with different intensity because of membrane destruction. These cardiomyocytes are usually characterized by irregular contractile activity and short life span. Later damaged cells maximally shrink acquiring a spherical shape (Fig. 1, *c*) and die. However, directly after cell separation in suspension we can see during several minutes rhythmically contracting cardiomyocytes in a massive of silent cells; these cells later quite down. Presumably, their contractile activity is caused by mechanical stimulation, which they experience during resuspending.

Structural integrity of cardiomyocytes isolated by the proposed method was confirmed by electron microscopy. Microphotographs clearly demonstrate typical structural organization of a cardiomyocyte. Sarcolemma, mitochondria, sarcoplasmic reticulum, and myofibrils have a characteristic appearance (Fig. 2). Orderly organization of sarcomers and clear-cut Z-bands are seen, actin myofilaments are parallel to myosin (Fig. 2, *a*). Heterogeneous structure of the cell membrane is seen in areas of desmosomes and tight junctions (Fig. 2, *b*). Oval nucleus is situated almost in the center of the cell, with clearly seen normally disposed nuclear pores on its membrane (Fig. 2, *c*). Myofibrils are divided by sarcoplasmic reticulum and mitochondria (Fig. 2, *d*), are rather compactly packed, and virtually completely fill the intracellular space.

The results evidence normal structure of isolated cells and recommend the above method for sparing separation of heart tissue into individual cells and operations with them for 6-8 h.

REFERENCES

1. S. Armstrong, J. M. Downey, and Ch. E. Ganote, *Cardiovasc. Res.*, **28**, 72-77 (1994).
2. G. Bkaily, N. Sperelakis, and J. Doane, *Am. J. Physiol.*, **247**, No. 6, Pt. 2, H1018-H1026 (1984).
3. H. Ichikawa, D. J. Hearse, and W. A. Coetzee, *Ibid.*, **266**, No. 6, Pt. 2, H511-H520 (1994).

4. G. Isenberg and U. Klockner, *Pflugers Arch.*, **395**, No. 1, 19-29 (1982).
5. K. Khrapko, N. Bodyak, W. G. Thilly, *et al.*, *Nucleic Acids Res.*, **27**, No. 11, 2434-2441 (1999).
6. A. Moscona and H. Moscona, *J. Anat.*, **86**, No. 3, 287-301 (1952).
7. T. Powell and V. W. Twist, *Biochem. Biophys. Res. Commun.*, **72**, No. 1, 327-333 (1976).
8. H. Satoh, L. A. Blatter, and D. M. Bers, *Am. J. Physiol.*, **272**, No. 2, Pt. 2, H657-H668 (1997).
9. R. S. Vander Heide, D. Rim, C. M. Hohl, and C. E. Ganote, *J. Mol. Cell. Cardiol.*, **22**, No. 2, 165-181 (1990).
10. B. C. Yang, D. S. Zander, and J. L. Mehta, *J. Pharmacol. Exp. Ther.*, **291**, No. 2, 733-738 (1999).