

ISOLATION, STAGES OF DIFFERENTIATION,
AND LONG TERM MAINTENANCE OF ADULT RAT MYOCYTES

Ronald E. Weishaar and Robert U. Simpson

Department of Pharmacology, The University of Michigan,
Medical Science I, Ann Arbor, Michigan 48109

ABSTRACT

Although a variety of techniques have been developed to isolate myocytes from adult hearts, the long term viability of such cells has only recently been investigated. In addition, relatively little is known about the stages of differentiation such cells proceed through following isolation. In the present study myocytes were isolated using two techniques, one involving retrograde perfusion via the aorta, and the other involving mechanical "shearing." In addition, several modifications were made to minimize the trauma normal associated with isolating myocytes from adult hearts. Both techniques yielded a high percentage of rod-shaped, quiescent myocytes, although myocytes isolated using the "shearing" method were less likely to remain viable for more than 24 hours. With both techniques those cells which remained viable for more than 24 hours proceeded through an identical pattern of differentiation leading to stable, attached cells which remained viable for up to four weeks. These results demonstrate that with the appropriate isolation techniques it is possible to maintain adult myocardial cells in culture for lengthy periods of time.

INTRODUCTION

Isolated cells maintained in culture represent a unique approach to examining important physiological responses in an environment in which the influence of neurotransmitters, hormones, and various pathological conditions can be discretely manipulated. Although cultured cells have been extensively employed to characterize metabolic events in a number of cell types, including adipocytes (Elks and Manganiello, 1984), fibroblasts (Goldstein et al., 1979), and transformed malignant cells (Thomas and Simpson, 1986), comparable studies with isolated adult myocytes maintained in culture for extended periods of time have proven difficult. This difficulty is associated with the intolerance of cardiac cells to the calcium-free conditions normally used for the isolation of a primary culture (Dow et al., 1978). Several

techniques have been employed to minimize such damage, including the addition of dimethylsulfoxide (Clark et al., 1978), and taurine, creatine and amino acids (Kao et al., 1980) to the isolation medium. Variations in the length of calcin-free exposure (Dow et al., 1981), and the osmolality of the isolation medium (Vahouny et al., 1970; Dow et al., 1981), have also been shown to influence the percentage of viable cells.

The present study compares two novel approaches to minimizing the tissue damage which accompanies preparation of primary cultures of adult myocytes, and also documents the differentiation process through which these cells progress during the three to four week period which they were maintained in culture.

MATERIALS AND METHODS

Primary cultures of adult myocytes were obtained using a modification of the retrograde heart perfusion method previously described by Bihler et al. (1984) (technique I), or by a similar procedure described by Jacobson (1977), in which isolated cells are harvested by gentle physical disruption (technique II). Adult male Sprague-Dawley rats weighing 300 to 400 grams were used for both procedures. Animals were heparinized (1000 U/100 gm body weight) thirty minutes prior to sacrifice. In both procedures attempts were made to minimize cellular damage by use of a depolarizing buffer containing 30 mM KCl during the calcium-free phase, and by inclusion of carnitine, taurine and saturating levels of essential amino acids and vitamins throughout the isolation and differentiation phases. In addition, the effects of incremental restoration of calcium to the medium during the isolation phase was also evaluated.

Technique I: After removal from the chest cavity the heart was rapidly blotted in 70% ethanol and perfused for 15 minutes via the aorta using an oxygenated (95% O₂, 5% CO₂) buffer composed of the following: 112 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂·2H₂O, 1.0 mM MgCl₂, 9.6 mM NaH₂PO₄, 24.0 mM NaHCO₃, 11.0 mM glucose, 5.0 mM carnitine, 60 mM taurine, saturating levels of essential amino acids, 75 U/ml penicillin and 50 µg/ml streptomycin, pH 7.4 (equilibration buffer I). The perfusate and the perfusion apparatus were sterilized prior to use, and sterile conditions were maintained through the isolation. After this equilibration period, the heart was depolarized by raising the concentration of KCl in the perfusate to 30 mM. MgCl₂ and glucose concentrations were increased to 3.4 and 15.0 mM, respectively, and the concentration of NaCl was reduced to 87 mM (depolarization buffer I). Two minutes later calcium-free perfusion was begun

(depolarization buffer I containing no CaCl_2). Exactly five minutes later, the concentration of CaCl_2 was raised to 0.025 mM, and 0.1% hyaluronidase and 0.1% collagenase (type II) were included (disruption buffer I). The heart was perfused in this manner for 12 minutes, after which it was removed from the perfusion apparatus, minced, and the tissue mince gently stirred using a magnetic stirring bar in an Erlenmeyer flask containing 10 ml of disruption buffer I. At five minute intervals for the next 20 minutes the supernatant was removed and the mince resuspended in disruption buffer I. The supernatant were centrifuged ($80 \times g$) and resuspended in equilibration buffer I containing 1.0% bovine serum albumin (BSA) and 0.1 mM CaCl_2 . The resuspended cells were centrifuged as before and resuspended in equilibration buffer I containing 1.0% BSA and 0.5 mM CaCl_2 . The cells were then centrifuged a third time, resuspended in equilibration buffer I containing 10% fetal calf serum, 1.2 mM CaCl_2 and insulin/transferrin/selenium, transferred to cultured flasks and incubated at 37°C. After two hours and again after 24 hours, the unattached cells were transferred to fresh culture flasks. This latter procedure prevented contamination with rapidly attaching fibroblasts.

Technique II: After sacrifice, 3-4 hearts were blotted in 70% ethanol and the apical portion dissected and cut in a staggered fashion to produce an "accordion-like" arrangement as described in detail by Jacobson (1977). The tissue was then suspended in a buffer composed of the following: 90 mM NaCl, 30 mM KCl, 5.6 mM glucose, 42 mM sucrose, 2.1 mM NaHCO_3 , 2.0 mM HEPES, 2.0 mM carnitine, 5.0 mM taurine, saturating levels of essential amino acids and vitamins, 75 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin (equilibration buffer IIA). The tissue was then transferred to a disruption vessel previously described by Jacobson (1977) and Sperelakis (1984), in which the opposing movements of thin teflon bristles produces a gentle shearing effect on the tissue. The tissue was suspended in equilibration buffer IIA containing 0.25% trypsin, and the teflon bristles rotated at 7 rpm. After 15 minutes the supernatant was removed and discarded, and replaced with the equilibration buffer IIA containing 0.1% collagenase. At 15 minute intervals for the next 105 minutes the supernatant was removed, centrifuged and the cells resuspended in the following buffer: 120 mM NaCl, 5.0 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 11.0 mM glucose, 2.0 mM carnitine, 5.0 mM taurine, saturating levels of essential amino acids in vitamins, penicillin and streptomycin (equilibration buffer IIB). The resuspended cells were then layered over 5 ml of calcium-free Joklik's modified essential medium (JMEM) containing 6% BSA and centrifuged as before. The lower three ml were then withdrawn and

diluted into JMEM containing 10% fetal calf serum, 1.2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.0 mM carnitine, 5.0 mM taurine, insulin/transferin/selenium and saturating levels of amino acids and vitamins. The cells were then transferred to tissue culture flasks and cultures were treated as before.

RESULTS

Roughly 50% of the cells obtained using Technique I were rod-shaped. These cells excluded trypan blue and were mostly quiescent. A larger percentage of the cells obtained using Technique II (roughly 75%) were rod-shaped, extruded trypan blue and were mostly quiescent. The remaining cells in both preparations were round and rapidly took up trypan blue. Those rod-shaped cells which did beat spontaneously immediately after isolation usually constricted into a round shape within one to two hours after isolation. Although a larger portion of rod-shaped cells were initially observed following isolation using Technique II, by 24 hours post-isolation, the percentage of viable rod-shaped cells remaining was less than 10-15%. By contrast, a larger percentage of the cells obtained using Technique I remained rod-shaped at 24 hours (roughly 25%). In both cases, the cells which were rod-shaped at 24 hours proceeded through a discrete series of differentiation states, resulting ultimately in attached, binucleate cells. This progression is described below and is illustrated in Figures 1-7.

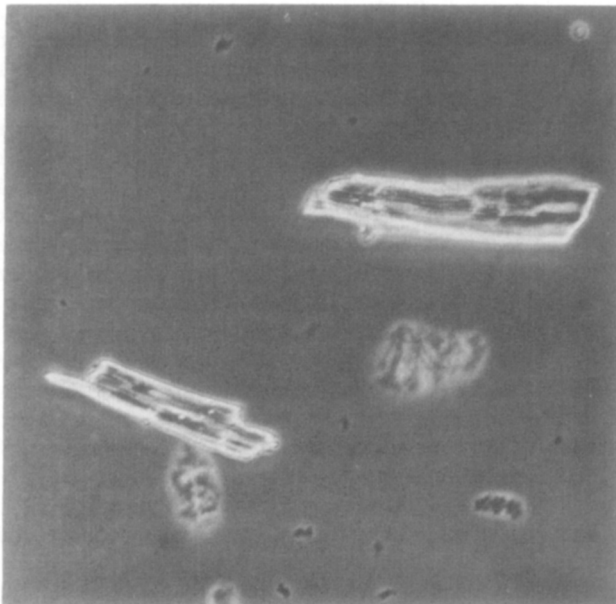


Figure 1. Myocytes one hour post-isolation.

Figure 1 shows myocytes one hour post-isolation. The appearance of the myocytes at this stage, and at all subsequent stages, is identical whether they are isolated using Technique I or Technique II.

At 2-4 days post-isolation, the edges of the rod-shaped cells begin to round (Figure 2), and by 4-8 days the cells are completely spherical (Figure 3).

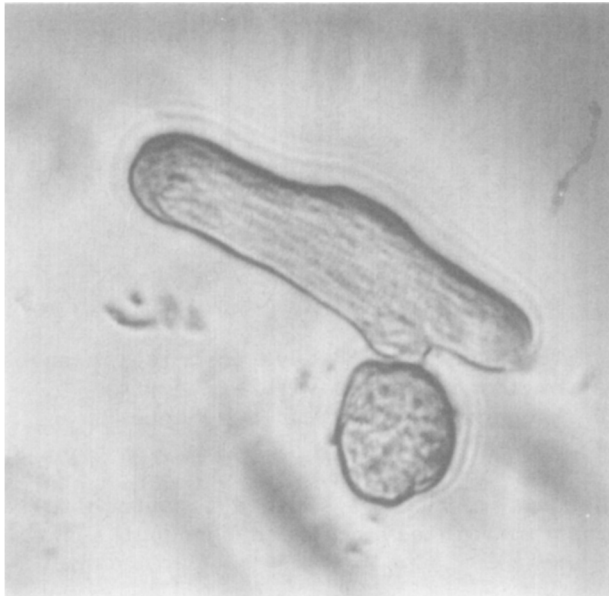


Figure 2. Myocytes 2-4 days post-isolation.



Figure 3. Myocytes 4-8 days post-isolation.
Arrow (↑) indicates viable myocyte.
Dark object is non-viable myocyte.

Shortly thereafter, the cells begin to attach to the culture flasks, and almost immediately begin to send out several projections (Figure 4).

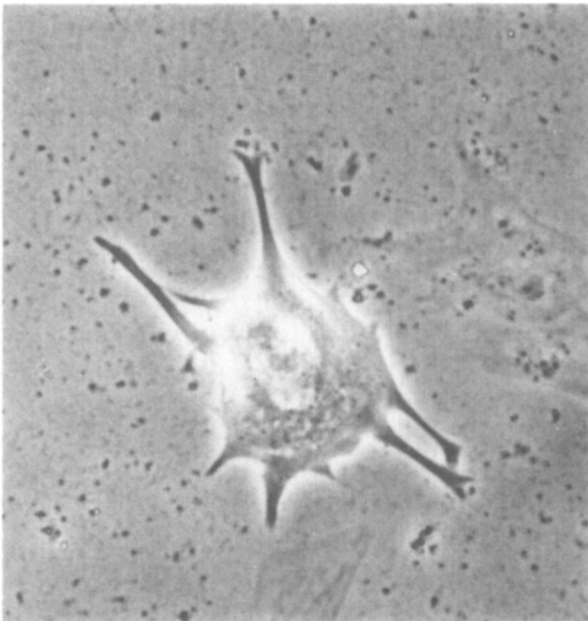


Figure 4. Myocyte shortly after attachment.

Although finger-like initially, many of the longer projections are lost by 8-10 days post-isolation, leaving a dense mesh of short projections surrounding the cell (Figure 5).

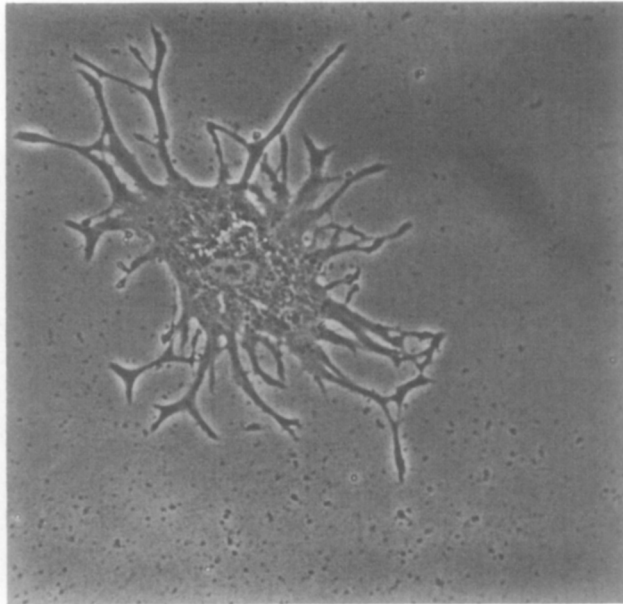


Figure 5. Myocyte 8-10 days post-isolation.

The cells also begin to flatten at this stage, and two nuclei are apparent. The number of projections then begins to decrease, until finally only 4 or 5 thicker primary projections remain (Figure 6).

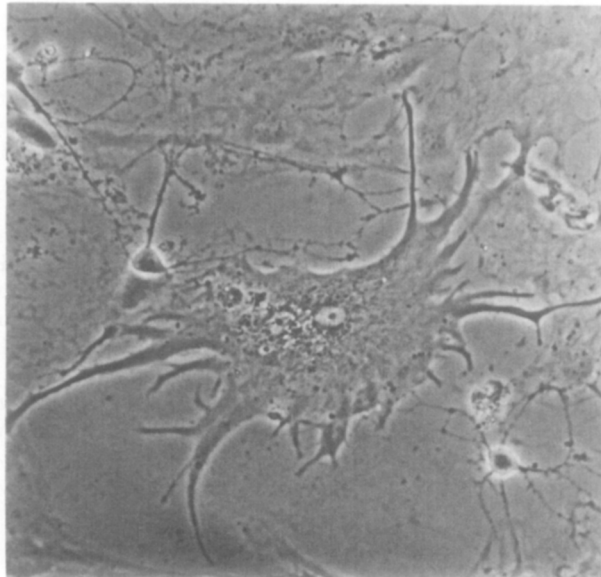


Figure 6. Mature myocyte 10-21 days post-isolation.

At this stage roughly 75-80% of the cells are observed to beat spontaneously, albeit asynchronously. No further obvious differentiation is observed for the next two weeks. At 3-4 weeks, a number of vacuoles are observed within the cells (Figure 7). The number of vacuoles within the cells increases steadily, and in most instances vacuoles are also noted outside of the cells. The cells begin to disintegrate shortly thereafter.

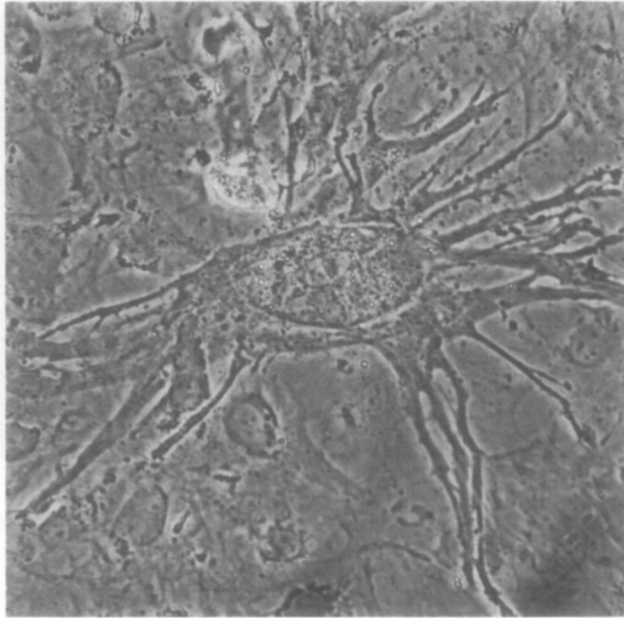


Figure 7. Degenerating myocyte, 3-4 weeks post-isolation.

DISCUSSION

This communication describes several modifications of existing isolation procedures designed to increase the yield of myocytes which remain viable for lengthy periods of time in culture. Although differences were found between the retrograde perfusion method (technique I) and the teflon bristle "shearing" method (technique II) regarding the yield of rod-shaped cells obtained, with both techniques cells which remained viable and rod-shaped after 24-48 hours went on to attach and then to differentiate through the same stages. Since the attached cells did not appear to undergo further differentiation following attachment and flattening, and remained viable for up to three weeks in culture, such cells should prove useful in evaluating a variety of key physiological, pharmacological and pathological events in cardiac cells.

This work was supported by the American Heart Association.

REFERENCES

- Bihler, I., Ho, T.K. and Sawh, P.C. (1984) Isolation of Ca^{2+} tolerant myocytes from adult rat heart. Canadian Journal of Physiology and Pharmacology, 62, 581-588.
- Bkaily, G., Sperelakis, N. and Doane, J. (1984) A new method for preparation of isolated single adult myocytes. American Journal of Physiology, 247, H101C-H1026.
- Clark, M.G., Gannon, B.J., Bodkin, N., Patten, G.S. and Berry, M.N. (1978) An improved procedure for the high-yield preparation of intact beating heart cells from the adult rat. Biochemical and morphologic study. Journal of Molecular and Cellular Cardiology, 10, 1101-1121.
- Dow, J.W., Harding, N.G.L. and Powel, T. (1981) Isolated cardiac myocytes. I. Preparation of adult myocytes and their homology with the intact tissue. Cardiovascular Research, 15, 483-514.
- Elks, M.L. and Manganiello, J.C. (1984) Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3',5'-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. Endocrinology, 115, 1262-1268.
- Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proceedings of the National Academy of Science, U.S.A., 76, 333-337.
- Jacobson, S.L. (1977) Culture of spontaneously contracting myocardial cells from adult rats. Cellular Structure and Function 2, 1-9.
- Kao, R.L., Christman, E.W., Luh, S.L., Krauhs, J.M., Tyers, G.F.O. and Williams, E.H. (1980) The effects of insulin and anoxia on the metabolism of isolated mature rat cardiac myocytes. Archives of Biochemistry and Biophysics, 203, 507-599.
- Thomas, G.A. and Simpson, R.U. (1986) High performance liquid chromatography analysis of 1,25-dihydroxy vitamin D_3 receptor in malignant cells. Correlation of effects on cell proliferation and receptor concentration. Cancer Biochemistry and Biophysics, 45, 221-224.
- Vahouny, G.V., Wei, R., Starkweather, R. and Davis, C. (1970) Preparation of beating heart cells from adult rats. Science, 167, 1616-1618.