A RAPID TECHNIQUE FOR THE ISOLATION AND PURIFICATION OF ADULT CARDIAC MUSCLE CELLS HAVING RESPIRATORY CONTROL AND A TOLERANCE TO CALCIUM

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SUMMARY

A rapid technique is described for the isolation of muscle cells from adult rat myocardium using in vitro perfusion with calcium-free bicarbonate buffer containing crude collagenase. Under optimum conditions, 5×10^6 cells per g tissue are obtained and the suspension may be purified to contain 70% intact cells. The complete procedure is rapid and isolated myocytes beat, exclude vital stains, have conventional sub-cellular morphology, show tight respiratory coupling and also have a tolerance to external calcium not found in cells isolated by other perfusion techniques. This represents a significant advance in the development of an isolated cell model for the study of myocardial mechanisms.

INTRODUCTION

Several methods have been reported for the isolation of beating cells from adult rat myocardium, including mechanical disruption (1-3), tissue incubation with proteolytic enzymes (4, 5) and organ perfusion using calcium-free media containing trypsin or collagenase with or without hyaluronidase (6-11). In only two cases were cell yields reported (5, 8) and in all other studies it was stated that cells rounded up immediately on exposure to solutions containing calcium chloride. Furthermore, only two groups (8, 11) have noted that preparations generally contain rod-shaped (intact) cells together with round cells which are damaged myocytes. Reported here is a technique for the isolation of muscle cells from adult rat heart which is rapid, produces a high cell yield and has almost 70% of the cell population in the form of intact myocytes. These muscle cells show tight respiratory coupling and are not hypersensitive to calcium.

MATERIALS AND METHODS

Female Sprague-Dawley rats were used weighing approximately 230 g. All reagents were 'Analar' grade or equivalent. Crude collagenase (Type 1, Cl. histolyticum) was obtained from Worthington. Pentex Fraction V bovine serum albumin from Miles Laboratories and Type F (Essentially Fatty Acid Free) from Sigma.

Cell Preparation: A rat was killed by cervical dislocation, the heart removed quickly and placed in ice-cold Krebs-Ringer bicarbonate buffer of the following composition (mmol/l): NaCl, 120.5; $NaHCO_3$, 13.1; KCl, 2.6; KH_2PO_4, 1.18; $MgSO_4$, 1.18; Glucose, 11.1. The heart was transferred to a tared beaker containing cold buffer, weighed, then mounted on the cannula of a standard perfusion column and chamber (12) containing 50 ml of buffer supplemented with 0.1% (w/v) type F albumin (KRBGA). Perfusion pressure was 70 mm Hg, temperature 36.8 $\pm$ 0.1 ^{o}C and pH was maintained at 7.40 $\pm$ 0.05 by gassing with 95% O_2/ 5% CO_2.

The organ was perfused until clear of blood and the buffer used for this initial washing procedure collected. An equal volume (approximately 10 ml) of KRBGA containing 25 μmol/l $CaCl_2$ was added to 20 mg crude collagenase. After a further 4 minutes perfusion with recirculation of the remaining buffer, this enzyme solution was added to the system and the coronary flow rate adjusted to 4-5 ml/min. Perfusion was then continued for a period of 16.5 minutes per g heart wet weight.

At the end of the perfusion period the heart was reweighed and then cut into two halves, which were chopped by a mechanical tissue cutter (13) at a blade interval setting of 1mm. The tissue was placed then in a teflon beaker containing 20 ml of gassed perfusate to which had been added sufficient type F albumin for a final concentration of 2% (w/v). Incubation of the tissue slices continued at 35 ^{o}C until dispersion was achieved by gentle agitation through a 10 ml serological pipette. After this time, generally 10-15 minutes, the suspension was filtered through 250 μm mesh nylon gauze and centrifuged for 1 minute at 22g. The cell pellet was washed once by resuspension and centrifugation at 20 ^{o}C in 20 ml of albumin-free buffer for 1 minute.

The washed cells (20 ml) were then layered in 10 ml aliquots each on to 40 ml of 4% (w/v) albumin (Fraction V) KRBGA and centrifuged at 22 g for 1 minute (20 ^{o}C). Supernatants were withdrawn and 20 ml of suspension in albumin-free buffer again layered on 4% (w/v) KRBGA and recentrifuged. The final cell pellets were resuspended in 20 ml 2% (w/v) KRBGA.

Cell counts were made in a bright line haemocytometer and cellular respiration rates determined by a conventional polarographic oxygen electrode (14).

RESULTS

Table I indicates cell yields obtained in a series of 19 experiments. At a mean buffer osmotic pressure of 260.7 mOsm/kg. mean heart weight increased by

TABLE I

Data from heart perfusion experiments

Buffer Osmotic Pressure (mOsm/kg)	Initial Heart Weight (g)	Post-Perfusion Weight Gain (%)	Rod Cells per g tissue (10^6)	
260.7	1.00	54.5	5.01	: Mean
0.90	0.03	3.55	0.30	: SEM
1.42	13.9	23.5	26.4	: CV%
(n=17)	(n=19)	(n=13)	(n=19)	

SEM = Standard Error of Mean: CV% = Coefficient of Variation

TABLE II

Sedimentation of Cell Suspensions

Post-Dispersal		1st Fractionation		2nd Fractionation		
10^6 Rod Cells per g tissue	% Total	10^6 Rod Cells per g tissue	% Total	10^6 Rod Cells per g tissue	% Total	
5.01	43.4	4.02	60.8	2.98*	67.1	: Mean
0.30	1.70	0.26	1.59	0.20	1.38	: SEM
26.4	15.2	27.1	11.1	29.9	8.7	: CV%
(n=19)		(n=18)		(n=19)		

*Recovery of 60% based on Mean Values.

54.5% during perfusion and 5.01 x 10^6 rod cells were obtained per g tissue wet weight. Higher osmotic pressures resulted in increasing numbers of dense and heavily granulated rod cells, whereas perfusions with heart weight gains above

70% or below 40% produced either increasing numbers of damaged (round) cells or low total cell yields.

The mean initial post-perfusion percentage of rod cells was 43.4%, which increased to 60.8% after one fractionation and reached 67.1% in the final suspension (Table II). The fractionation procedure resulted in the elimination of cellular debris, red blood cells and endothelial cells, and almost 60% recovery of the initial rod cells was achieved. Figure 1 shows the typical appearance of a purified preparation. Virtually all of the rod cells excluded the vital stains trypan blue and lissamine green, whereas round cells did not. When examined under the electron microscope, rod cells showed morphology similar to that found in the intact tissue. Cell dimensions varied from widths of 10 to 35 μm and lengths of 80 to 150 μm.

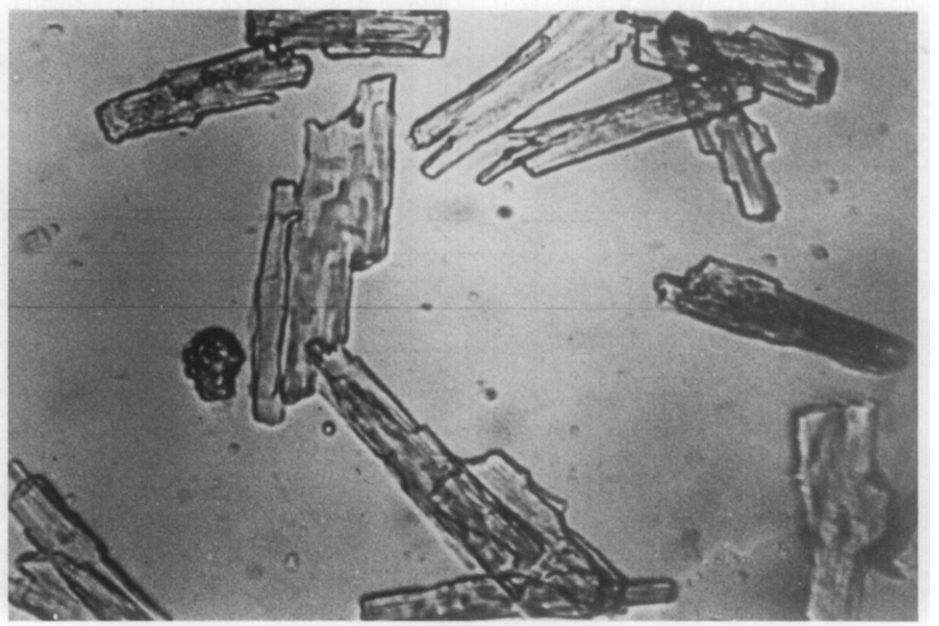

Figure 1 Light micrograph of myocyte suspension after complete preparation and purification procedure (Magnification x 400).

Enriched rod cell preparations incubated for 45 minutes at 37 °C in 2% (w/v) KRBGA containing either trace amounts or 0.3 mmol/l calcium chloride showed no change in composition, within the statistics of cell counting. Cells incubated in 1 mmol/l $CaCl_2$ showed a reduction in total rod cell count of 10% after 15 minutes incubation and 25% after 45 minutes. In calcium-free medium approximately 30% of the rod cells were beating, whereas over 75% beat when placed in media containing 1 mmol/l calcium chloride. Beating cells have been observed in suspension more than 24 hours after preparation. Round cells do not show contractile activity.

Table III gives data on the stimulation of rod cell suspension oxygen consumptions by 2 μg/ml of the uncoupler FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone), 1 μg/ml of the potassium ionophore

TABLE III

Stimulation of Cellular Oxygen Consumptions

FCCP (2μg/ml)	Valinomycin (1μg/ml)	Na-Succinate (10 mmol/l)	
(n = 10)	(n=7)	(n=5)	
5.32	3.90	3.22	: Mean
0.55	0.29	0.43	: SEM
32.5	19.7	30.1	: CV%

Linear basal respiration rates were established before addition of stimulant in 5 μl or 10 μl volumes. 1 ml of cell suspension used at 32 °C. Equivalent solvent volumes were run as blanks and calibration was achieved by conventional methods (14). Values for stimulations listed in the table obtained from the ratio of the respiration rate with stimulant to that of the preceding control period.

valinomycin and by sodium succinate (10 mmol/l). From the FCCP experiments a mean value of 5.32 is obtained for the Respiratory Control Index of these cells. Valinomycin stimulated respiration by 3.90 and succinate by 3.22. In addition, it was found that cyanide (3 mmol/l) inhibited respiration completely and rotenone (5 μg/ml) produced partial inhibition. Cellular respiration was linear with time in all samples for at least six hours after preparation. In contrast, enriched round cell preparations recovered from the supernatants of the sedimented fractions were not stimulated by either FCCP or valinomycin. Succinate did stimulate in a variable manner, but the response was never greater than 25% of that seen in the enriched rod preparation.

DISCUSSION

The initial post-perfusion yield of rod cells at 5.01×10^6 per g tissue wet weight is some 5-10 times higher than the only other published data for isolated myocytes (5) using prolonged incubation of tissue fragments with collagenase. By the technique described here, some 3.0×10^6 rod cells per g tissue wet weight at 70% purity are obtained within approximately 90 minutes from the start of perfusion, whereas tissue incubation methods require four 20 minute periods merely to achieve digestion (5). The inclusion of EGTA in perfusion media can increase the initial proportion of rod cells to over 90%, but on incubation with calcium chloride (0.5 mmol/l) at 37 ^{o}C this reduces to 4% (11). Myocytes in this preparation show a tolerance to external calcium which has not been reported previously in studies using organ perfusion.

Respiratory control is one of the most sensitive indicators of mitochondrial integrity (15) and the FCCP experiments confirm tight respiratory coupling of the enriched myocyte preparations. Stimulation by valinomycin suggests that mitochondrial potassium conductance is also low, similar to that found for isolated liver cells (16).

Thus, isolated muscle cells prepared by the present technique have typical cross-striations, beat, exclude vital stains and have conventional morphology. In addition, intact cells show respiratory coupling and a tolerance to exogenous calcium chloride. These factors support the conclusion that this preparation should prove a useful model for biophysical and biochemical investigations concerning myocardial mechanisms at the cellular level.

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