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Stability of the contractile assembly and Ca^{2+}-activated tension in adenovirus infected adult cardiac myocytes

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Abstract

Adenovirus-mediated gene transfer into adult cardiac myocytes in primary culture is a potentially useful method to study the structure and function of the contractile apparatus. However, the consequences of adenovirus infection on the highly differentiated state of the cultured myocyte have not been determined. We report here a detailed analysis of myofilament structure and function over time in primary culture and after adenovirus infection. Adult rat ventricular myocytes in primary culture were infected with a recombinant adenovirus vector expressing either the *LacZ* or alkaline phosphatase reporter gene. Control and infected myocytes were collected at days 0–7 post-isolation/infection, and myofilament isoform expression was determined by SDS-PAGE and Western blot. Laser scanning densitometry showed that the α- to β-myosin heavy chain ratio, the stoichiometry of the myosin light chains and the expression of the adult troponin T isoform did not change over time in culture or with adenovirus treatment. Importantly, examination of Ca^{2+}-activated tension in single myocytes showed no change in the shape or position of the tension-pCa relationship in the control and adenovirus infected myocytes during primary culture. These results indicate that the structure and function of adult cardiac myocytes are stable in short term primary culture and are not affected by adenovirus infection *per se*, and therefore provide the foundation for the use of adenovirus-mediated myofilament gene transfer to study contractile apparatus structure and function in adult cardiac myocytes. (Mol Cell Biochem **181**: 143–155, 1998)

Key words: cardiac muscle, contractility, myofilament, virus

Introduction

One important goal of researchers in the contractility field is to define the functional significance of individual protein subunits in the complex, multimeric contractile assembly in both the normal and diseased myocardium. Direct gene transfer into cardiac myocytes *in vivo* [14, 17] and *in vitro* [13] is one method that may allow a rapid dissection of the role of specific protein subunits in the context of the intact myocyte. Although direct injection of plasmid DNA into the adult heart *in vivo* is relatively inefficient [14], infection with recombinant adenovirus vectors is highly effective for introducing foreign genes into adult cardiac myocytes in primary culture [13]. Important questions, however, on the specificity of this approach have not been fully addressed. For example, the stability of the contractile assembly of adult cardiac myocytes over time in culture and the possible nonspecific effects of adenovirus-mediated gene delivery on myocyte stability in culture have not been well characterized.

It is well known that, over time in culture, adult ventricular myocytes exhibit dedifferentiation as characterized by cell spreading, loss of rod-shaped morphology and re-expression of fetal protein isoforms. This dedifferentiation process is affected by various components in the medium, including fetal bovine serum [29], and thus can be significantly slowed by maintenance of the adult myocytes in the absence of

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serum [6, 23, 25, 29]. Even in serum-free culture conditions, however, there is disagreement on the number of days adult cardiac myocytes are stable in culture. Nag *et al*. [23] reported that within one week there was evidence of spreading in adult rat cardiac myocytes cultured in serum-free medium, whereas Volz *et al*. [29] reported that rod-shaped morphology was maintained for 15 days in culture. In another study, rod-shaped morphology was maintained in cultured adult rat cardiac myocytes for 6–8 days under serum free conditions, however there was an increase in β-myosin heavy chain (MHC) mRNA expression and a decrease in α-MHC mRNA expression indicating re-expression of a fetal gene in the adult cardiac myocytes, another hallmark of dedifferentiation [6]. Thus, the duration of primary culture may significantly influence the stoichiometry of the myofilament proteins. Changes in cell morphology and/or stoichiometry are important because they will have profound effects on myocyte contractile function [22]. One important purpose of the present study, therefore, was to examine cardiac myocyte contractile apparatus structure at multiple time points, using myocyte morphology, MHC and troponin T (TnT) isoform expression, and myosin light chain (MLC) 1 and 2 stoichiometry as indexes of the differentiated state of cardiac myocytes cultured in serum-free conditions.

A second major purpose of this study was to determine whether recombinant adenovirus-mediated gene transfer into adult cardiac myocytes in primary culture affects cardiac myocyte structure by examining, as described above, myocyte morphology, MHC and TnT isoform expression, and MLC stoichiometry. Adenovirus infection of adult cardiac myocytes results in highly efficient expression of a *LacZ* reporter gene without apparent changes in the viability or cell number of rod-shaped myocytes 72 h post isolation [13]. Because several key myofilament proteins such as troponin I, troponin T and myosin heavy chain have half-lives in the range of 3–5 days [19], adult ventricular myocytes in primary culture must maintain their morphology, isoform expression and protein stoichiometry for longer times (i.e. 5–7 days) after infection to provide a stable background for structure-function analysis of a transferred myofilament gene. Thus, in the present study, cultured adult cardiac myocytes were examined for changes in cellular morphology, MHC and TnT isoform expression, and MLC stoichiometry over a 7 day period after exposure to a recombinant adenovirus.

Ultimately, the usefulness of myofilament gene transfer into adult cardiac myocytes in primary culture as a strategy for studying the functional significance of a gene product in the myofilament assembly depends on demonstrating that the Ca^{2+}-activated contractile properties of the cardiac myocyte are stable during the culture period. Earlier studies have examined aspects of cardiac contractility by measuring the magnitude and rate of myocyte shortening and relengthening in response to electrical stimulation [1, 6]. This assay, while useful, does not directly report the force generating capacity of the contractile machinery in the isometric state. Direct measurement of Ca^{2+}-activated isometric tension under controlled activating conditions over a range of days in primary culture would therefore be of value. In addition, these measurements would provide a rigorous test of the stability of the contractile apparatus due to the acute dependence of Ca^{2+}-activated tension generation on myofilament isoform composition and stoichiometry [22]. Therefore, a final purpose of this study was to examine Ca^{2+}-activated isometric tension development in single adult cardiac myocytes at multiple time points in primary culture. This detailed analysis of myofilament structure and function over time in culture and following reporter gene transfer *in vitro*, serves as a foundation for the implementation of myofilament gene transfer into cultured adult cardiac myocytes for structure/function analysis.

Materials and methods

Primary cultures of ventricular myocytes

Female Sprague-Dawley rats weighing 200 g were injected with sodium heparin (1000 units/kg) and euthanized 15 min later by i.p. injection of sodium pentobarbital (50–100 mg/kg body weight). The heart was removed, mounted on a modified Langendorff perfusion apparatus, and perfused with Krebs-Henseleit (in mmol/L, 118 NaCl, 4.8 KCl, 25 HEPES, 1.25 K_2HPO_4, 1.25 $MgS0_4$, 11 glucose) buffer (KHB) containing 1 mM $CaCl_2$ for 5 min followed by perfusion with Ca^{2+}-free KHB (no added Ca^{2+}). After 5 min, 0.5 mg/ml collagenase (Type II, Worthington, Freehold, NJ, USA) and 0.2 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA) were added to the Ca^{2+}-free KHB perfusate and the heart was perfused for 15 min with 60 ml of recirculating perfusate. The Ca^{2+} concentration in the perfusion solution was brought to 1 mM in three 200 µl additions of a 100 mmol/L $CaCl_2$ stock solution at 30 sec intervals [12]. After 15 min, the heart was taken down, the atria were removed, and the ventricles were cut open and gently minced in 20 ml of 1 mmol/L Ca^{2+}-KHB/enzyme perfusion solution in a sterile beaker. The minced ventricles were incubated in this digestion solution at 37°C for 10 min with gentle swirling. The initial 20 ml aliquot of digestion solution was removed and discarded, and replaced with 20 ml of remaining digestion solution, and incubated at 37°C for 15 min with gentle swirling. The digestion solution was collected and centrifuged in a clinical centrifuge at 45 × g for 30 sec. The cells were resuspended in 1 mmol/L Ca^{2+}-KHB + 2% BSA. The digestion and resuspension steps were repeated once more, and the cells from the two digests were combined. The solution was titrated to 1.75 mmol/L Ca^{2+} by adding $CaCl_2$ in 250 µmol/

L aliquots at 5 min intervals. After the last $CaCl_2$ addition, the cells were centrifuged briefly, the supernatant was removed, and the cells were resuspended in DMEM + 5% fetal bovine serum (FBS) + 50 units/ml penicillin and 50 μg/ml streptomycin (P/S). The myocytes were plated on laminin-coated tissue culture plates (Collaborative Biomedical Products, Bedford, MA) at ~100 myocytes/mm^2 and placed in a 37°C, 5% CO_2 incubator. After 2 h the media was removed and replaced with serum-free DMEM + P/S, and the dishes were returned to the incubator. Culture medium was replaced with fresh serum-free DMEM + P/S every 2–3 days.

Transfection of myocyte cultures

Twenty-four hours after plating onto laminin-coated 35 mm wells, ventricular myocyte cultures were transfected with the plasmid pCMVβGal by one of the following methods. Myocyte survival 24 h post transfection was determined by counting attached rod-shaped myocytes using a light microscope, and transgene expression was determined by histochemical staining 24–48 h post transfection.

DEAE-dextran
Standard DEAE-dextran protocol was followed [26]. One hour before transfection, media was changed to DMEM + P/S containing 20 mmol/L 2,3-butanedione monoxime (BDM) [4] or maintained in DMEM + P/S. Myocytes were treated with 0.5,1.0, or 5.0 μg pCMVβGal + 400 μg DEAE-dextran in tris buffered saline for 1 h followed by exposure to 100 μM chloroquine for 1 h. Myocytes treated with BDM were maintained in BDM-containing medium for 24 h after transfection, and then returned to DMEM + P/S. Alternatively, myocytes were treated with 0.5–7.0 μg pCMVβGal + 80 or 400 μg DEAE-dextran + 15-3000 PFU/cell AdCMVβAhAP (described below) for 1.5 h [9]. After transfection, cultures were washed twice with PBS and maintained in DMEM + P/S.

Poly-L-ornithine
Myocytes were maintained in DMEM + P/S or switched to DMEM + P/S containing 20 mmol/L BDM 1 h prior to transfection. Control and BDM wells were treated with 0.0005–50 μg/ml poly-L-ornithine + 5 μg pCMVβGal/well for 6 h after which the cells were shocked with 10% DMSO for 2 min and then returned to the appropriate medium, DMEM + P/S +/– BDM [5]. Alternatively, the myocytes were treated with 0.0005–1.0 μg/ml poly-L-ornithine + 5 μg pCMVβgal/well ± 2000 PFU/cell AdCMVβAhAP for 6 h, washed twice with PBS, and maintained in DMEM + P/S.

LipofectAMINE (Gibco-BRL, Grand Island, NY)
Myocytes were switched to DMEM (no P/S) or DMEM + 20 mmol/L BDM (no P/S) 1 h prior to transfection. Myocytes were treated with a transfection mixture consisting of 2 μg pCMVβGal + 2, 4, 6, 8 μl lipofectAMINE ± 2000 PFU/cell AdCMVβAhAP diluted in DMEM or DMEM + BDM for 5 h. Wells were washed with PBS and fed with DMEM or DMEM + BDM for an additional 5 h, and then maintained in DMEM + P/S.

Calcium phosphate
Myocytes were maintained in DMEM + P/S or switched to DMEM + P/S + 20 mmol/L BDM 1 h before transfection. The calcium phosphate precipitate was prepared as described [26]. Precipitate was added to the media and incubated for 5 h, washed twice with PBS, and fed with DMEM + P/S or DMEM + P/S + BDM, incubated for 4 h and then maintained in DMEM + P/S.

Electroporation
1×10^6 freshly isolated myocytes were suspended in either Ca^{2+}-free KHB or Ca^{2+}-free KHB + 20 mmol/L BDM containing 20 μg pCMVβgal and transferred to an electroporation cuvette (0.4 cm gap). Myocytes were pulsed at 1.2 kV, 25 μF or 0.25 kV, 960 μF, plated onto laminin coated dishes (1 cuvette/35 mm well) in Ca^{2+}-free KHB ± BDM overnight, then Ca^{2+} was titrated to 1.75 mmol/L before changing media to DMEM + P/S.

Adenoviral infection

The replication-deficient adenoviral constructs, AdCMVlacZ, AdRSVlacZ, andAdCMVβAhAP, are derived from adenovirus serotype 5 and were kindly provided by Dr. Beverly Davidson from the University of Michigan. Expression of the bacterial *LacZ* gene in vectors AdCMVlacZ and AdRSVlacZ [16] is driven by either the cytomegalovirus immediate early promoter (CMV) or the long terminal repeat of the Rous sarcoma virus (RSV). The vector AdCMVβAhAP [24] contains the CMV enhancer and β-actin promoter driving expression of the human alkaline phosphatase gene. The titers of AdCMVlacZ, AdRSVlacZ, and AdCMVβAhAP were 2.4×10^{10} plaque forming units (PFU)/ml, 1.2×10^{11} PFU/ml, and 2×10^{10} PFU/ml respectively.

Cultures were infected 5–24 h after plating by replacing the medium with serum-free DMEM + P/S containing the desired dose (PFU/ml) of adenovirus. Adenovirus doses ranged from 2.4×10^4 PFU/ml to 2.4×10^8 PFU/ml. After 24 h, the medium was replaced with DMEM + P/S and cultures were examined for expression of the transgene 24 h to 8 days after infection.

Histochemical staining

Detection of *LacZ* gene expression in cardiac myocytes was accomplished by histochemical staining for β-galactosidase

(β-gal) activity. Myocyte cultures were washed twice with PBS (in mmol/L, 8.9 Na_2HPO_4, 1.1 NaH_2PO_4, 150 NaCl) and then fixed for 5 min using 2% formaldehyde, 0.2% glutaraldehyde in PBS. Fixative was washed off with PBS and Color Solution (in mmol/L, 5 $K_3Fe(CN)_6$ + 5 $K_4Fe(CN)_6$-$3H_2O$ + 2 $MgCl_2$ in PBS) containing 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added. Cells were stained for 6–12 h and then examined for β-gal positive blue cells. Detection of alkaline phosphatase gene expression was accomplished by histochemical staining using the Sigma Diagnostics Leukocyte Alkaline Phosphatase kit (Cat. # 85L-2) following the instructions provided. Briefly, myocytes were fixed for 30 sec in citrate buffered acetone, rinsed with deionized water, stained in an alkaline-dye mixture for 30 min, and then washed with deionized water for 2 min. Alkaline phosphatase positive cells stained dark blue.

SDS-PAGE and immunoblotting

For SDS-PAGE, samples containing approximately 10–20 cardiac myocytes were collected from tissue culture plates using silicone-treated micropipettes. Cells were viewed using the 10 × objective of an inverted light microscope. Rod-shaped myocytes were collected by touching with the silicone adhesive-treated micropipette tip. Collection of multiple cells with one micropipette was accomplished by dragging the tip across an area containing essentially all rod-shaped myocytes, which adhered to the adhesive and were then transferred to a microfuge tube. The myocytes were stored in 10 µl of SDS sample buffer at –20°C for SDS-PAGE and silver staining as described previously [21]. Samples were sonicated for 10 min and centrifuged just prior to gel analysis. The gel system had the following features: (1) acrylamide-piperazine diacrylamide ratio of 200:1; (2) pH of 9.3 in the running gel buffer; (3) molarity of the running gel buffer of 0.75 M. The stacking gel contained a 3.5% acrylamide and the separating gel contained 12.5% acrylamide. The gel was run at a constant current of 20 mA for 4–5 h, fixed in 10% glutaraldehyde overnight and then washed in deionized water for several days before silver staining. Silver-stained gels were scanned using a LKB densitometer and the areas under the peaks were integrated to determine relative expression of protein isoforms.

Western blots were carried out as described previously [31], using the anti-striated muscle TnT monoclonal antibody JLT-12 (Sigma; 1:100) for 2 h. Primary antibody was detected by peroxidase conjugated goat anti-mouse IgG (Sigma; 1:1000) and an enhanced chemiluminescence detection assay (Amersham, Arlington Heights, IL, USA). The number of myocytes loaded per lane was 800–2000.

Experimental chamber and apparatus for mechanical studies

The chamber used for myocyte force measurements was 6 × 6 × 3/4 inches, and was constructed out of stainless steel and aluminum. The chamber has 4 troughs that were milled out of the stainless steel section (volume per trough approximately 200 µl) which provide wells for attaching, and relaxing and activating the myocyte. A force transducer (Cambridge Technology, Watertown, MA, USA; Model 403A; sensitivity, 5 µg; 1–99% response time, 1 msec) and moving coil galvanometer (Cambridge Technology 6350 optical scanner) were attached to the chamber via three-way positioners to allow their exact positioning. The temperature of the experimental chamber was controlled using thermo-electric modules (Melcor Inc., Trenton, NJ, USA) that were coupled to a recirculating water bath heat-sink. The chamber was positioned on an anti-vibration table.

Cardiac myocyte attachment procedure

Acutely isolated myocytes

The myocyte attachment procedure was adapted from an earlier study [27] that has been described in detail previously [20], and briefly reviewed below. Micropipettes, coated with a silicone adhesive (Dow Chemical, Midland, MI, USA) and connected to the recording devices, were positioned over and then attached to a single rod-shaped cardiac myocyte. An attached myocyte was permeabilized in relaxing solution containing 0.2% Triton X-100 for approximately 1 min as longer treatment (to 30 min) does not alter the tension-pCa relationship [20, 27].

Cardiac myocytes in primary culture

After enzymatic isolation of ventricular myocytes, 2×10^4 cells were plated on to laminin-coated glass cover slips and placed in six-well culture dishes. After 1–6 days in primary culture the cover slips were removed from the dishes, and the DMEM was replaced using several washes of relaxing solution (see below). Single, rod-shaped, cardiac myocytes were then attached to the glass micropipettes as above. For all cardiac myocytes used in the mechanical studies, sarcomere length was set at 2.20 µm by viewing the myocyte using light microscopy and then adjusting the overall length of the preparation by way of three-way translators.

Activating and relaxing solutions

Relaxing and activating solutions contained (in mmol/L): 7 EGTA, 1 free Mg^{2+}, 4 MgATP, 14.5 creatine phosphate, 20 imidazole, and sufficient KCl to yield a total ionic strength

of 180 mmol/L. Solution pH was adjusted to 7.00 with KOH. The values for MgATP, free Mg^{2+}, pH etc. were derived from the literature for intact mammalian striated muscle fibers as described earlier [11]. The pCa (i.e.-log [Ca^{2+}]) of the relaxing solution was set at 9.0, while the pCa of the solution for maximal activation was 4.5. The computer program of A. Fabiato [8] was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, employing the stability constants listed by Godt and Lindley [10]. The apparent stability constant for Ca^{2+}-EGTA is corrected for ionic strength, pH, and experimental temperature (15°C).

Measurement of steady-state isometric tension-Ca^{2+} relationship

At each pCa, steady-state isometric tension was allowed to develop, after which the fiber was rapidly (< 0.5 msec) slackened to obtain the tension baseline. The myocyte was then relaxed. The difference between developed tension and the tension baseline following the slack step was measured as total tension. To obtain active tension, resting tension measured at pCa 9.0 was subtracted from total tension. The myocyte was transferred to relaxing solution after each activation at a given pCa. Tension-pCa relationships were constructed by expressing tensions (P) at various submaximal Ca^{2+} concentrations as fractions of the tension at maximal activation, P_o (i.e. isometric tension at pCa 4.5), that bracketed the submaximal activations.

Data acquisition, curve fitting and statistics

Data acquisition

Data were acquired using a Nicolet storage oscilloscope (model 310).

Curve fitting

To derive values for the Hill coefficient (n) and mid-point (termed pCa_{50} or K) from the tension-pCa relationship data was fit using the Marquardt-Levenberg nonlinear least squares fitting algorithm using the Hill equation in the form: $P = [Ca^{2+}]^n/(K^n + [Ca^{2+}]^n)$, where P is tension as a fraction of maximum tension (P_o), K is the [Ca^{2+}] that yields one-half maximum tension, and n_H is the Hill coefficient.

Statistics

Analysis of variance (ANOVA) was used to examine whether significant differences in myofilament isoform composition, myofilament stoichiometry, pCa_{50}, or n_H resulted during primary culture. A probability level of $p < 0.05$ was selected as indicating significance.

Results

Gene transfer into adult cardiac myocytes in primary culture

Attempts to transfect adult cardiac myocytes by several different methods including DEAE-dextran with and without adenovirus, lipofection, electroporation, poly-L-ornithine with and without adenovirus and calcium phosphate were unsuccessful (results not shown). These methods uniformly caused severe damage to the cardiac myocytes, leaving no surviving cells at 24 h post transfection. When myocytes were exposed to low levels of poly-L-ornithine or transfected in the presence of 2,3-butanedione monoxime (BDM), an inhibitor of contraction [4], myocytes survived transfection, as evidenced by maintained rod-shaped morphology, however, there was no expression of the transgene indicating that gene transfer did not occur (results not shown). Thus, although there are reports that neonatal cardiac myocytes may be transfected by various methods, these techniques appear to be ineffective for adult cardiac myocytes.

Kirshenbaum *et al*. [13] have shown that adenovinus vectors provide highly efficient gene transfer into ventricular myocytes cultured in serum-free conditions at 48 h post infection, without adversely affecting the cellular morphology of the myocytes. In the present study, we have expanded on these results by examining infection, using adenovirus vectors with three different promoters, of the adult cardiac myocytes in serum-free culture conditions for up to 8 days in primary culture.

The efficiency and stability of gene transfer into adult ventricular myocytes cultured in serum-free conditions was determined in myocytes infected with AdCMVlacZ or AdRSVlacZ 24 h after isolation, and histochemically stained for β-galactosidase activity 24–48 h later. Cultures were studied by light microscopy and were scored by counting the β-gal positive rod-shaped myocytes/total rod-shaped myocytes. Cells with extensive pseudoprocess formation or spreading at the ends were not considered to be rod-shaped. Cultures infected with 2.4×10^4 to 2.4×10^8 PFU/ml of AdCMVlacZ or 2.4×10^5 to 2.4×10^8 PFU/ml of AdRSVlacZ showed increasing expression of the LacZgene with increasing dose of adenoviral vector (Fig. 1). The dose of AdCMVlacZ and AdRSVlacZ required for half-maximal *LacZ* gene expression was 4×10^6 and 6×10^7 PFU/ml respectively. At doses greater than 10^8 PFU/ml, 90% of the myocytes were β-galactosidase positive. These results are similar to those of Kirshenbaum *et al*. [13] who detected infection in 80% of myocytes at 2×10^6 PFU and 90% at 2×10^8 PFU with the vector AdHCMVsp1 LacZ. Because the CMV promoter was more efficient than the RSV promoter,

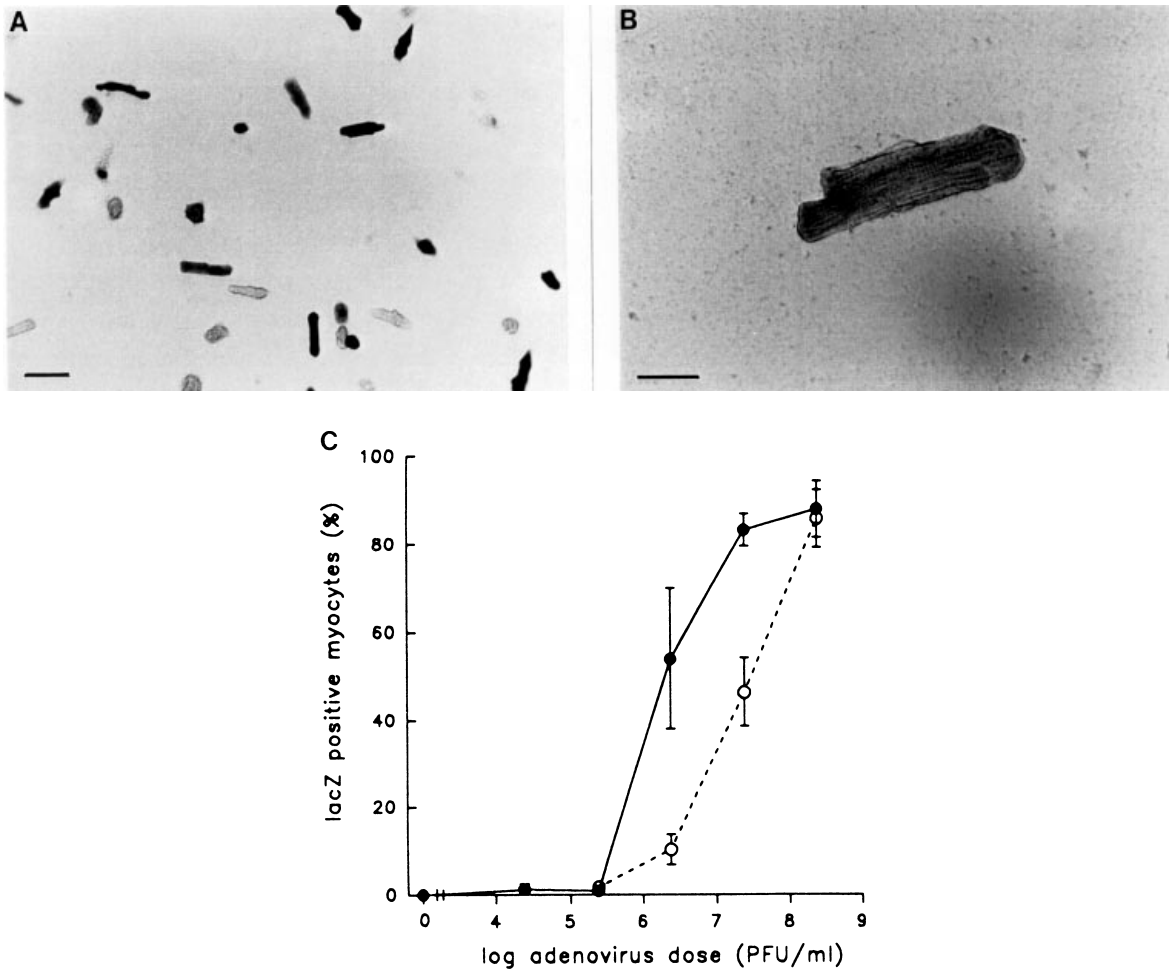

Fig. 1. Efficiency of adenovirus infection of adult cardiac myocytes in primary culture. After 24 h in culture, myocytes were infected with AdCMVlacZ at 2.4×10^4–2.4×10^8 PFU/ml or with AdRSVlacZ at 2.4×10^5–2.4×10^8PFU/ml. Histochemical staining for β-galactosidase activity was done 24–48 h post infection. (A) and (B) Light photomicrographs showing β-gal positive staining in cardiac myocytes. Bar is 100 μm; (C) Summary of infection efficiency of AdCMVlacZ (filled circles, solid line) and AdRSVlacZ (open circles, dashed line) in rod-shaped cardiac myocytes. Results are given as mean ± S.E.M. (about 4,000–20,000 rod-shaped cardiac myocytes scored/point).

all subsequent studies were performed using either the AdCMVlacZ or AdCMVβAhAP adenovirus vectors.

It was unknown whether transgene expression would continue beyond 48 hours post infection *in vitro*. Long term expression of the transgene was examined by infecting myocyte cultures with 1×10^7 PFU/ml AdCMVβAhAP 4–6 h after isolation and then staining 48 h to 8 days later to determine if gene expression was stable. Histochemical staining showed that 86–88% of the myocytes expressed the alkaline phosphatase gene at 48, 72, and 96 h post infection, and that ~99% of the myocytes expressed the transgene at 8 days post infection (Table 1). These results indicate that efficient and stable gene transfer and expression is obtained using recombinant adenovirus to infect isolated ventricular myocytes.

Stability of cardiac myocytes in primary culture

Another important concern in studies using gene transfer into cultured adult ventricular myocytes is that the adult

Table 1. Adult cardiac myocytes infected with AdCMVβAhAP stably expressed high levels of the transgene over time in culture

Time post infection (h)	% alkaline phosphatase positive rod-shaped cardiac myocytes
48	86.6
72	88.5
96	87.0
192	98.8

Positive staining was scored by counting rod-shaped myocytes which exhibited blue/purple staining through the 10 × objective of a Nikon light microscope.

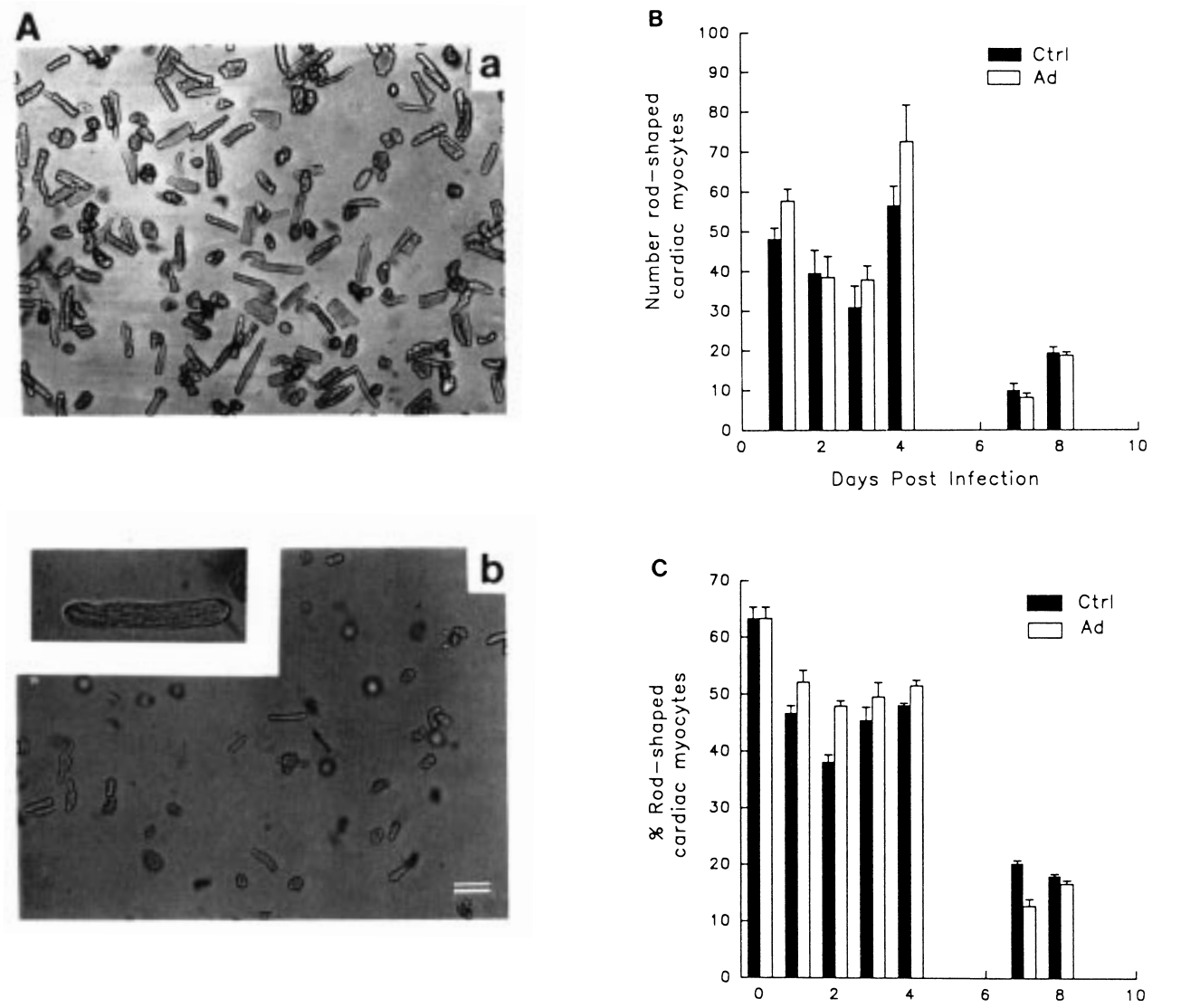

Fig. 2. Effects of adenovirus infection on the morphology of cardiac myocytes in culture. (A) Light photomicrographs showing representative fields at 24 h (a) and 6 days (b) post isolation. Arrow indicates rod-shaped myocyte (enlarged in inset). Bar is 100 μm; (B) Average number of rod-shaped myocytes/field over time in primary culture. Cells were fixed before counting in 2% formaldehyde, 0.2% glutaraldehyde in PBS as described in Materials and methods. Each field had an area of 2.43 mm^2, and 7–20 fields were counted from each culture well. Results are given as the average #rods/field ± S.E.M. (n = 3 wells); (C) Average % of rod-shaped myocytes/field over time in culture. Results are given as average number of rod-shaped cells/total number of cells per field ± S.E.M. (n = 3 wells, fields are as described for part (B).

ventricular myocytes undergo marked dedifferentiation as they adapt to culture conditions. Dedifferentiation is characterized by loss of rod-shaped cellular morphology, sarcomere disarray, expression of fetal contractile protein isoforms [6], and decreased contractile function [1]. In the present study, the stability of primary cultures of control and adenovirus-infected adult ventricular myocytes was assessed by studying the maintenance of rod-shaped morphology and MLC stoichiometry, the expression of the adult MHC and TnT isoforms and the ability to produce force upon Ca^{2+} activation. Myocytes were exposed to serum for only 2 h to aid in attachment [25], after which the serum was removed and the medium was maintained serum-free, since the presence of fetal bovine serum in the culture medium has been implicated in increasing or stimulating dedifferentiation in primary cultures of cardiac myocytes [29]. Control cultures were not exposed to adenovirus, and infected cultures were treated with 1×10^7 PFU/ml of AdCMVlacZ or AdCMVβAhAP. The number of rod-shaped myocytes were counted as described above and compared to the numbers of fibroblasts, rounded up cells, and spreading myocytes. The number and percent of rod-shaped myocytes

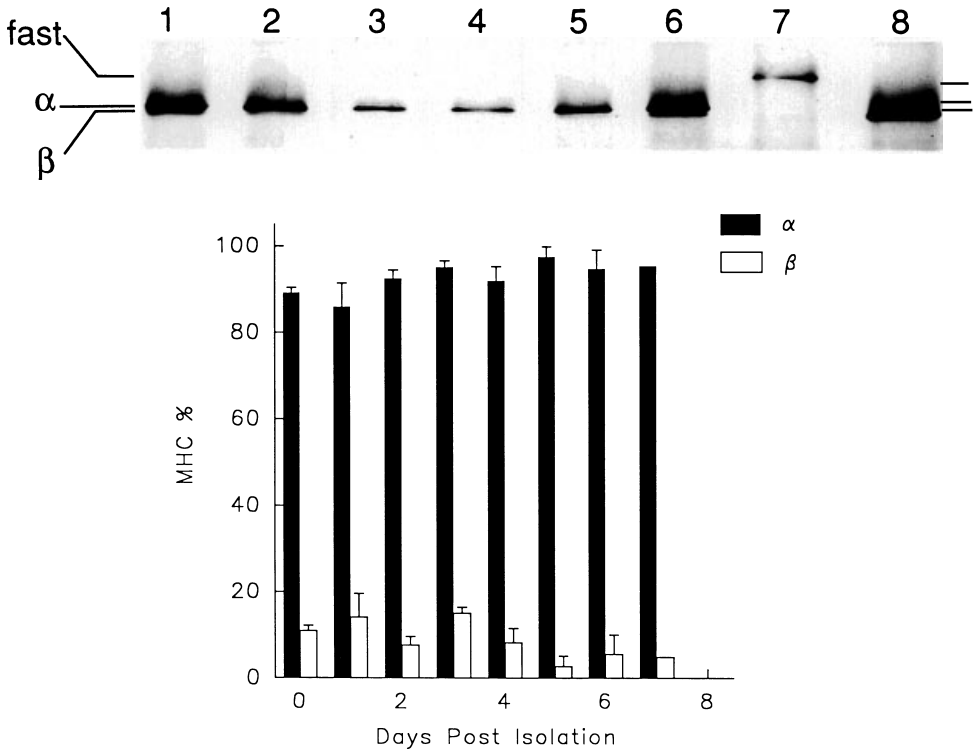

Fig. 3. Stability of myosin heavy chain (MHC) isoform expression in primary ventricular myocyte cultures. (Upper part). Representative SDS-polyacrylamide gel of uninfected cardiac myocytes collected at various times after isolation. Lanes are: 1: 0 h; 2: 24 h; 3: 48 h; 4: 72 h; 5: 96 h; 6: 0 h; 7: psoas (fast skeletal muscle); 8: soleus (slow skeletal muscle). The number of cardiac myocytes loaded per lane was ≈10–20, and this variation accounts for differences in silver staining intensity among the lanes. (Lower part). Summary of cardiac MHC isoform expression in cultured myocytes. α and β MHC isoforms are shown as percent of total MHC as determined by densitometry of silver-stained gels. Results are given as mean ± S.E.M. (n = 2–26; n = 1 for day 7).

per field decreased similarly in both control and adenovirus-treated cultures over time (Fig. 2), indicating that infection with adenovirus does not adversely affect the morphological dedifferentiation of cardiac myocytes in primary culture.

Further assessment of the differentiated state of the cardiac myocytes in culture was performed by examining the isoform expression pattern of two contractile proteins, MHC and TnT. Rod-shaped cardiac myocytes were collected at various time points in culture and contractile proteins were analyzed by gel electrophoresis (Figs 3 and 4. Differences in silver staining intensity between lanes can be accounted for by variations in loading, see figure legend.). Densitometric analysis of silver-stained gels showed that there was no change in the ratio of α- to β-MHC in either control or adenovirus infected myocytes (Figs 3 and 4). A transition from expression of the α-MHC to the β-MHC in adult rat cardiac myocytes in primary culture would indicate that fetal isoform expression had been induced and therefore that the myocytes were dedifferentiating. Because there is little information on expression of myofilament regulatory proteins during culture, we examined the expression pattern of troponin T (TnT), which is an important determinant of the Ca^{2+} sensitivity of force production in striated muscle [22]. Western blot analysis determined that the adult and not the embryonic isoform of cardiac troponin T was expressed in both control and adenovirus-infected cultures of ventricular myocytes (Fig. 5). Our results indicate that the rod shaped myocytes have retained their differentiated state in terms of MHC and TnT isoform expression.

The stoichiometry of myosin light chains also was quantified by densitometry of the silver stained gels. In rod-shaped myocytes collected at various time points from control and infected cultures, there was no change in the ratio of myosin light chains (MLC) 1 and 2 (Fig. 6), showing that the MLC stoichiometry was retained. Taken together these results support the conclusion that under the conditions described here, ventricular myocytes are stable in short term culture and are not adversely affected by infection with adenovirus.

Another important indicator of the myocyte's differentiated state is measurement of Ca^{2+}-activated isometric tension generation at different time points in primary culture. Because the tension-pCa relationship is very sensitive to myofilament isoform composition and stoichiometry [3, 22], it represents a direct test of the functional status of the contractile assembly in the context of the working cell. For

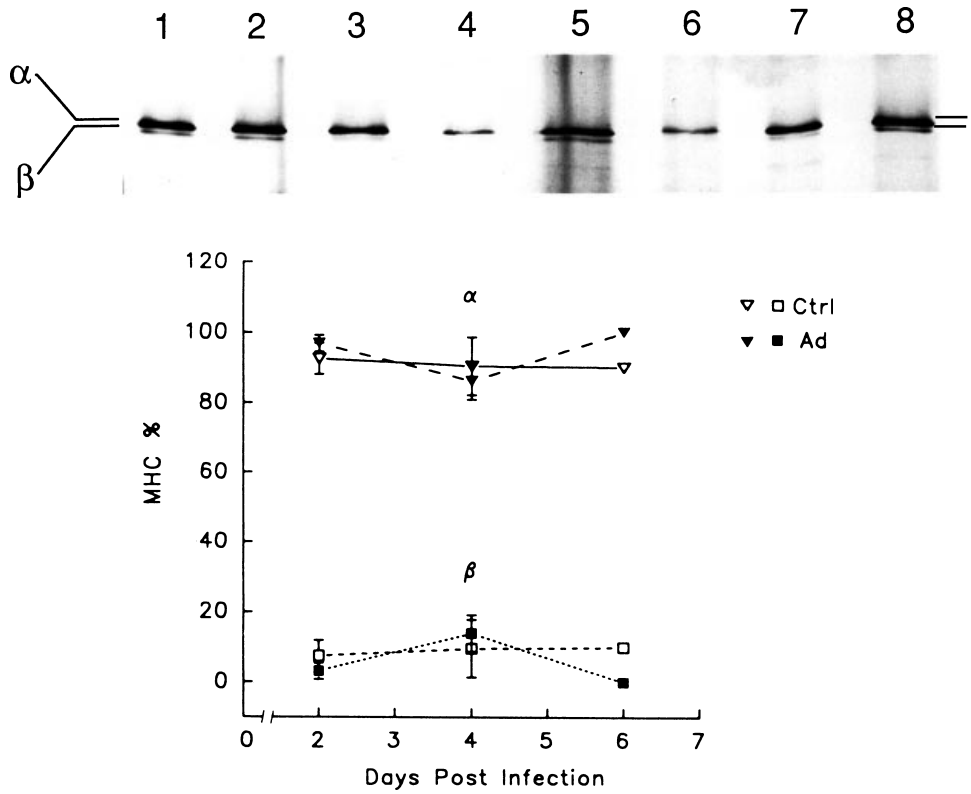

Fig. 4. Effects of adenovirus infection on MHC isoform expression in cardiac myocytes in primary culture. (Upper part). Representative SDS-polyacrylamide gel of infected and uninfected primary ventricular myocytes. The lane designations represent the time post isolation or infection at which the myocytes were collected. Lanes are: 1: 0 h control (C); 2: 48 h C; 3: 48 h adenovirus infected (Ad); 4: 96 h C; 5: 96 h Ad; 6: 144 h C; 7: 0 h C; 8: 24 h C. The number of cardiac myocytes loaded per lane was ≈10–20, and this variation accounts for differences in silver staining intensity among the lanes. (Lower part). Comparison of α and β MHC isoform expression in control and adenovirus infected ventricular myocytes, shown as percent of total. Rod-shaped myocytes were infected with adenovirus at 1×10^7 PFU/ml. Results are given as the mean ± S.E.M. (n = 2–15). In some cases the error bars were smaller than the symbol size.

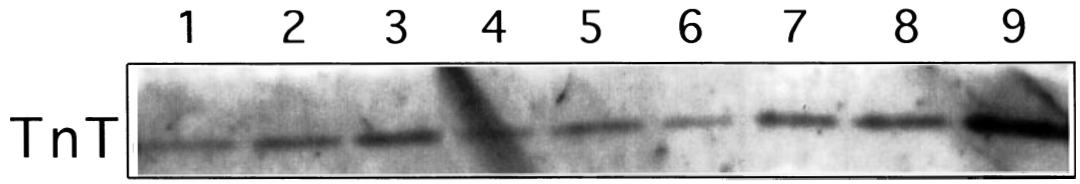

Fig. 5. Western blot analysis showing no change in TnT isoform expression within adult cardiac myocytes in primary culture. Samples were loaded with the following number of rod-shaped myocytes and culture durations, respectively. Lanes 1: 800, 72 h; 2: 1600, 72 h; 3: 2000, 72 h; 4: 800, 48 h; 5: 1600, 48 h; 6: 800, 72 h; 7: 1600, 72 h; 8: 2000, 72 h; 9: 800, freshly isolated.

example, small alterations in troponin C content that may be undetected in gel analysis may be possible to detect in this functional assay as evidenced by a rightward shift in the position of the tension-pCa relationship [3, 22]. Although measurements of tension development have been reported for acutely isolated single cardiac myocytes [7, 20, 27], this is the first report of Ca^{2+}-activated tension measurements on adult cardiac myocytes at multiple time points in primary culture. With these issues in mind, Ca^{2+}-activated tension was measured in cardiac myocytes cultured for up to 6 days. Figure 7 shows slow and fast time-base records of isometric tension from a single cardiac myocyte in primary culture. The tension responses to controlled Ca^{2+} activations in this and other cardiac myocytes obtained at 1–6 days in primary culture were obtained for comparison with data from acutely isolated myocytes (Fig. 8, Table 2). In some myocytes it was possible to estimate cross-sectional area (width and depth measurements) to calculate force per unit area. Normalized force was 31.3 ± 5.4, 33.5 ± 17, 17.9 ± 3.8, and 19.8 ± 6.2 at 0, 2, 3, and 6 days in primary culture respectively. There was no significant

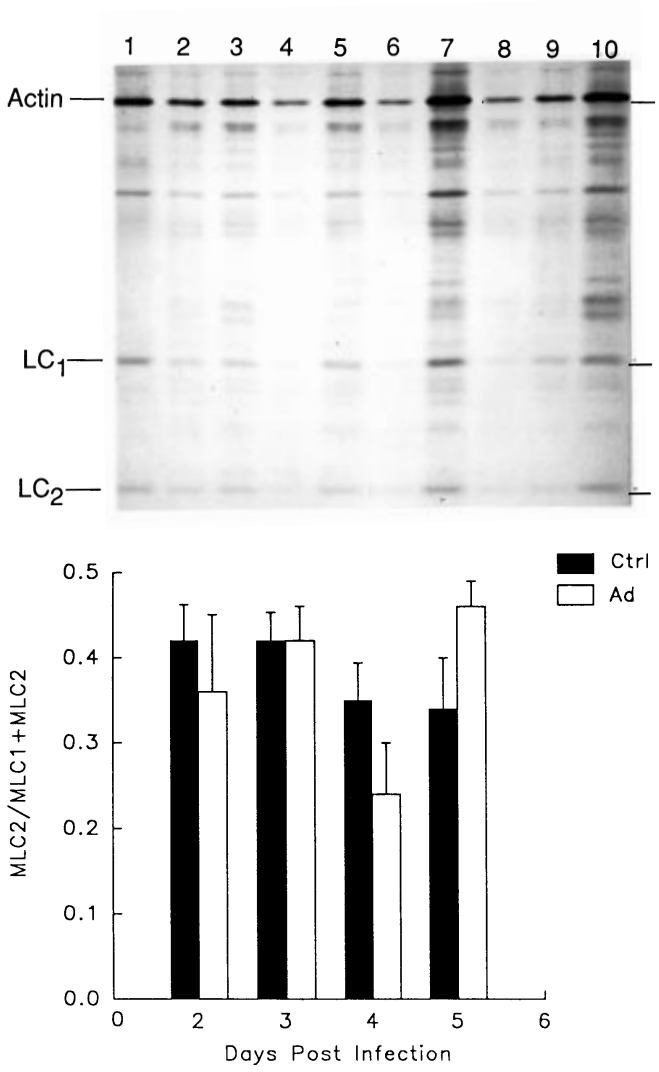

Fig. 6. Effect of adenovirus on the stoichiometry of the cardiac myosin light chains in ventricular myocytes in primary culture. (Upper part). Representative SDS-polyacrylamide gel of infected and uninfected primary ventricular myocytes. Lane designations represent the time post isolation or infection at which the ventricular myocytes were collected. Lanes are: 1: 0 h control (C); 2: 48 h C; 3: 48 h infected (Ad); 4: 72 h C; 5: 72 h Ad; 6: 96 h C; 7: 96 h Ad; 8:120 h C; 9: 120 h Ad; 10: 0 h C. The number of cardiac myocytes loaded per lane was ≈10–20, and this variation accounts for differences in silver staining intensity among the lanes. Analysis of several gels showed no consistent differences between control and adenovirus infected myocytes in terms of intensity of silver staining. (Lower part). Summary of the stoichiometry of the cardiac myosin light chains. Results are given as the mean ratio of MLC_2/MLC_1+MLC_2 ± S.E.M. (n = 2–6).

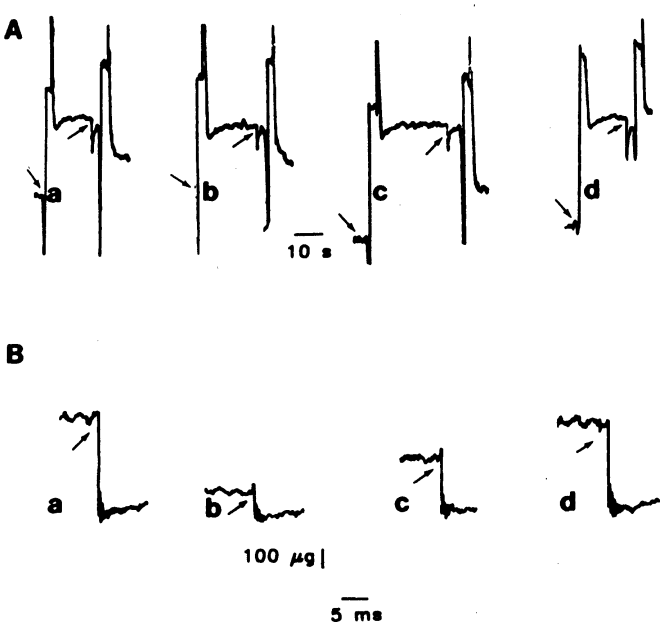

Fig. 7. Direct determination of Ca^{2+}-activated tension generation in a single cardiac myocyte at 48 h in primary culture. (A) Original slow time-base records of isometric tension. The pCa of the activating solution for records a–d were 4.5, 5.8, 5.4 and 4.5, respectively. Typically, each myocyte was activated using 6–8 different pCas, and only a subset of these activations are shown in the figure. For a–d, the first arrow indicates the transfer of the cardiac myocyte from relaxing solution to activating solution (tension spikes are due to the cardiac myocyte crossing the solution-air interface). At the second arrow cardiac myocyte length was rapidly released to obtain a tension baseline (see records in B) and transferred back to relaxing solution at which point the cardiac myocyte length was reextended; (B) Fast time-base records of isometric tension for the same single cardiac myocyte in part A. The solution pCa for records a–d were 4.5, 9.0, 5.8, 4.5, respectively. These records were used to determine the active tension generated at each pCa because the tension baseline in the slow time-base tension records shown in (A) are variable owing to small changes in surface tension surrounding the micropipettes upon translating the experimental chamber. Record b shows fast time-base record at pCa 9.0 to obtain resting tension which was then subtracted from the total tension value (e.g. a) to obtain the active tension generated by the myocyte at that particular pCa. Arrows indicate time point at which length change was introduced to obtain the tension baseline.

difference in normalized force over time in culture as assessed by one-way ANOVA. In addition, these values for normalized force are generally comparable to those reported earlier in acutely isolated single cardiac myocytes [27]. These results are evidence that the Ca^{2+}-activated force generating capacity of the myocytes is not significantly altered in culture, at least under these experimental conditions. Results further showed that the shape and the position of the tension-pCa relationship were not altered during primary culture. Specifically, the pCa_{50} and Hill coefficients (Table 2) were not significantly altered throughout the culture period as assessed by ANOVA. In other experiments the tension-pCa relationship was examined after infection of cardiac myocytes with a replication deficient, non-recombinant adenovirus to test whether adenoviral infection per se had any effects on Ca^{2+}-activated tension. Results showed that the shape and position of the tension-pCa relationship was not changed from the untreated myocytes (Fig. 8). These data provide strong functional evidence that the expression and stoichiometry of regulatory protein isoforms are not altered under these primary culture

Table 2. Summary of pCa_{50} and Hill coefficients obtained from single cardiac myocytes in primary culture

	Days in primary culture				
	0	1	2	3	6
pCa_{50}	5.74 ± 0.05	5.68 ± 0.05	5.75 ± 0.11	5.83 ± 0.06	5.76 ± 0.08
Hill coefficient	2.0 ± 0.3	1.8 ± 0.2	2.4 ± 0.5	3.0 ± 0.5	2.6 ± 1.0

Values are mean ± S.E.M. with average of four observations per group. No significant differences in pCa_{50} or Hill coefficients were seen at different days in culture as determined by one-way ANOVA.

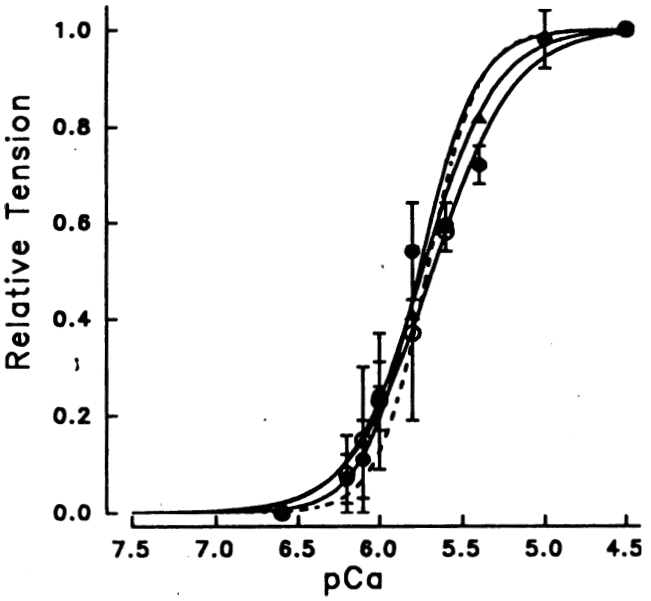

Fig. 8. Summary of tension-pCa relationships for single cardiac myocytes examined at different time points in primary culture and after infection with a non-recombinant adenovirus. Filled triangles are data from acutely isolated myocytes (day 0), open circles are day 1, filled circles are day 6, and the dashed line is non-recombinant JM-17-infected myocytes at day 3. Tension values are normalized to the maximum tension value obtained in each myocyte at pCa 4.5. Values are mean ± S.E. (n = 4). In cases where data overlapped directly only the filled circle data points are visible.

conditions, and that adenoviral infection per se does not alter the differentiated status of the cardiac contractile assembly. These functional results are consistent with the biochemical analysis of contractile and regulatory protein isoform composition and stoichiometry in adenoviral treated and untreated cardiac myocytes in primary culture.

Discussion

The results reported here provide an essential foundation for studies using adenovirus-mediated gene transfer into single adult cardiac myocytes for assessment of contractile structure and function. Specifically, control and adenovirus treated adult cardiac myocytes maintained their rod-shaped morphology, the stoichiometry of the MLCs, and MHC and TnT isoform composition in primary culture for at least 7 days. Adenovirus-mediated gene transfer was highly efficient and led to expression of the transgene in 87–99% of the rod-shaped myocytes over this time in culture. Importantly, functional assays showed that Ca^{2+}-activated tension development was unaffected by the duration of primary culture or by adenovirus infection.

There is little information in the literature on the stoichiometry and isoform composition of contractile regulatory proteins in cultured adult cardiac myocytes. It is important to examine these points because alterations in myofilament isoform composition or stoichiometry are known to have profound effects on the structure and function of the myocyte. For example, altering stoichiometry by partial extraction of MLC_2 leads to increased Ca^{2+} sensitivity of tension, whereas partial extraction of TnC decreases Ca^{2+} sensitivity of contraction in skeletal muscle fibers [22]. An altered stoichiometry of myofilament proteins may play a role in certain disease states, such as hypertrophic cardiomyopathy [28]. In addition, altered expression of other troponin subunit isoforms appear to have marked effects on Ca^{2+} sensitivity of tension ([22], and references therein). It was important, therefore, to characterize the stoichiometry and regulatory protein isoform composition of both control and adenovirus-infected myocytes in primary culture to establish the usefulness of this method for gene transfer of myofilament proteins into the cultured myocytes. In the present study, control and adenovirus-infected adult cardiac myocytes exhibited no changes in the α to β-MHC ratio, in the expression of the TnT isoforms, nor in the stoichiometry of the myosin light chains over 7 days in culture. These results establish that neither time in culture nor adenovirus-mediated reporter gene transfer affects the stability of the contractile protein stoichiometry and isoform expression in adult rat cardiac myocytes in primary culture.

It was also critical to evaluate the effect of adenovirus itself on the functional status of the contractile machinery over the culture period. In the present study, contractile function was determined directly in control and adenovirus-treated adult cardiac myocytes in primary culture by measuring Ca^{2+}-activated tension development. In a recent study, adenovirus-mediated gene transfer of a mutant β-MHC construct into cultured adult feline cardiac myocytes was reported to cause sarcomere disarray and myofilament disruption. However, an assessment of a possible functional deficit caused by the mutation could not be accomplished [18]. The demonstration here of direct analysis of Ca^{2+}-activated isometric tension generation in the context of a stable adult cardiac myocyte makes feasible the study of functional deficits resulting from the gene transfer of mutated myofilament proteins (e.g. [18]).

Presently, the primary genetic-based technique for studying contractile protein structure and function in the context of the intact myocyte has been the transgenic animal approach. This method offers a powerful approach as it allows evaluation of the transgene in the context of the organ *in vivo*, and permits study of developmental questions such as the timing and importance of myofilament isoform switching. Adenovirus-mediated gene transfer into adult cardiac myocytes in primary culture may be a powerful adjunct to transgenic mouse technology that could in principle permit rapid examination of the primary defect caused by altered gene expression. Since expression of the transferred gene is observed in the majority of the infected myocytes, uniform remodelling of the cultured myocytes is possible for proteins with a sufficiently rapid turnover rate. In fact, recently published data [30] demonstrates nearly complete remodelling of the contractile apparatus within seven days after adenovirus-mediated gene transfer of slow skeletal TnI into adult cardiac myocytes in primary culture. It must be recognized however that the study of myofilament proteins with longer half-lives than TnI, for example MLC_1 and MLC_2 [19], may be difficult using this method, at least under current conditions. Another limitation of the adenovirus approach is that the size of the insert is limited by the ability of the adenovirus to package only 105% of its genome size into infectious particles. Bett *et al*. [2] have recently described a system allowing packaging of up to 7.8 kb inserts and Marian *et al*. [18] have used this system to construct a recombinant adenovirus carrying a 7 kb β-MHC expression cassette. Another new adenoviral packaging system has been described [15], however, that allows replacement of large portions of the adenovirus genome with 25–30 kb of inserted DNA. Thus, adenovirus-mediated myofilament gene transfer is a potentially useful method to complement transgenic mouse models for the study of myofilament genes.

In summary, we have extensively characterized the cellular morphology, myosin heavy chain and troponin T isoform expression, myosin light chain 1 and 2 stoichiometry and Ca^{2+} sensitivity of tension development of adult rat cardiac myocytes in primary culture in the presence and absence of adenovirus infection. The results reported here provide the necessary foundation for contractile structure/function studies to be performed where the myofilament is remodelled after overexpression of one altered myofilament gene in the presence of an otherwise stable myofilament assembly.

Acknowledgements

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