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Isolation and Culture of Adult Mouse Cardiac Myocytes for Signaling Studies

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Abstract

The Alliance for Cellular Signaling (AfCS) has chosen cultured adult mouse cardiac myocytes as a system for studying cellular signaling. We developed a reproducible protocol for the isolation and culture of large numbers of viable, rod-shaped myocytes. With this protocol, we routinely isolated 1.5to 1.7 million myocytes per heart, of which 65% to 74% were rod-shaped. After 24 hours in culture, myocytes were 80% rod-shaped, and 88% of the viable, rod-shaped myocytes originally plated were retained. Cultured myocytes demonstrated predictable ligand-induced changes in accumulation of cAMP, phosphorylation of signaling proteins, and excitation-contraction coupling (both calcium transients and contraction). By these criteria, the short-term cultured adult mouse cardiac myocytes are suitable for signaling studies. For future studies requiring the expression of exogenous proteins, protein mutants, or vector-based RNA interference (RNAi), we established a long-term culture system (72 hours) that relies on medium supplemented with 2, 3-butanedione monoxime as well as insulin, transferrin, and selenium. Myocytes cultured for 72 hours in this manner remained viable and were approximately 70% rod-shaped. Finally, using adenovirus-mediated gene transduction, we demonstrated expression of an exogenous β-galactosidase reporter gene for 72 hours in cultured myocytes.

Introduction

The Alliance for Cellular Signaling (AfCS) is a consortium of laboratories and scientists dedicated to understanding the complexities of signal transduction. To study signaling, the AfCS has chosen two model cell systems, B lymphocytes and cardiac myocytes from the mouse. The basis for the selection of these two cell types is described elsewhere(1,2), as is the description of the cell culture system for mouse B lymphocytes (3). This manuscript describes the cell culture system for adult mouse cardiac myocytes.

Cardiac myocytes respond to a variety of hormonal, neural, mechanical, and electrical stimuli by altering both their force and rate of contraction. Stimulation of calcium influx through sarcolemmal calcium channels, and subsequent activation of calcium release from the sarcoplasmic reticulum, initiate contraction (excitation-contraction coupling) (4). Activation of G protein–coupled receptors, such as β-adrenergic receptors (coupled to Gs) or muscarinic receptors (coupled to Gi), regulate contractile force by modulating production of cAMP and activation of the cAMP-dependent protein kinase (PKA)(5,6,7). In addition, PKA-mediated phosphorylation of troponin I regulates contractile force, and phosphorylation of phospholamban, a regulator of the sarcoplasmic reticulum calcium pump, controls reuptake of calcium and relaxation(4,8,9).

Cardiac myocytes also undergo hypertrophy as a compensatory response to either physiological stimuli, such as exercise, or pathological stimuli, such as hypertension(10). The signaling pathways regulating hypertrophy encompass a wide array of signaling molecules and pathways, including G protein–coupled receptors, cytokine receptors, protein kinase C, phosphotidylinositol 3-kinase (PI3-kinase), MAP kinases, and calcineurin(11,12,13).

Although adult mouse cardiac myocytes are an intriguing model for the AfCS, the isolation of large numbers of these cells and their subsequent culture under physiological conditions is not routine; however, recent reports in the literature suggest that it is feasible(14,15,16). The challenge for the AfCS was to develop a protocol for the rapid and consistent isolation of large numbers of myocytes (4 to 5 million) at one time. In addition, isolated myocytes had to survive short-term culture (24 hours) without a significant loss of viable, rod-shaped myocytes. Myocytes in short-term culture had to be suitable for both biochemical studies, such as measurement of protein phosphorylation, and studies of excitation-contraction coupling, such as calcium transients and contraction. A long-term culture system capable of high efficiency gene transduction was also needed in order to manipulate expression of individual signaling proteins, such as by vector-based RNA interference (RNAi)(17) or by dominant-negative signaling proteins. Finally, the protocols for the isolation and culture of myocytes had to be reproducible among different laboratories.

This report describes protocols for the isolation and culture of adult mouse cardiac myocytes and for their short- and long-term culture. To determine if the cultured myocytes were suitable for signaling studies, ligand-induced changes in the accumulation of cAMP, in the phosphorylation of signaling proteins, and in excitation-contraction coupling were measured. Finally, myocytes were infected with an adenovirus-β-galactosidase reporter gene to demonstrate successful gene transduction and expression for 72 hours. These experiments provide the basis for future signaling studies in cardiac myocytes and, ultimately, the examination of how different signaling modules interact.

Results and Discussion

Isolation of Adult Mouse Cardiac Myocytes

For the isolation of adult mouse cardiac myocytes, we developed a protocol (PP00000125, PP00000126) based on established procedures in rat and rabbit, and we recently expanded the protocol to include mouse(14,15,16). Briefly, this protocol entails removing the heart, cannulating the aorta on a perfusion system, arresting the heart with a retrograde perfusion of calcium-free buffer, dissociating myocytes using a collagenase-based enzymatic solution, and reintroducing calcium to produce isolated, quiescent, rod-shaped myocytes (Table 1 is available in the accompanying PDF file). Using this protocol, we routinely obtained 1.5 to 1.7 million myocytes per heart, of which 65% to 74% were rod-shaped (Table 2). The total myocyte yields were approximately two- to three-fold higher than previously published reports for the isolation of mouse myocytes(14,15,16). Similar results were obtained in both the Laboratory for the Development of Signaling Assays (LDSA) and the Cell Preparation and Analysis Laboratory (CPAL) (Table 2), demonstrating that the procedure was transferable between laboratories. Further, myocytes were isolated from multiple hearts (up to four), permitting the isolation of up to six million myocytes for a single experiment.

We identified several critical factors in the protocol that improved myocyte isolation (summarized in Table 1). First, using isoflurane, an inhalation anesthetic, significantly improved myocyte yields compared to using a combination of ketamine and xylazine. Anesthetics like ketamine reduce respiratory rate and have a long onset period. This increases the risk of ischemia due to respiratory depression, which can reduce myocyte yields. Second, including 2, 3-butanedione monoxime, a contractile inhibitor, in the perfusion buffer improved yields by preventing myocyte contracture during the isolation procedure. Third, perfusion buffer pH was maintained with HEPES, and, contrary to expectations, we found no beneficial effect on myocyte yield of oxygenating the perfusion buffer. Fourth, liberase blendzyme, a recombinant enzyme mix containing collagenase and other proteases (Roche Molecular Biochemicals, Indianapolis, IN), supplemented with trypsin was used to digest hearts. Crude collagenases are the conventional choice of enzyme, and we had very good results with different collagenase preparations (collagenase type II, Worthington Biochemicals, Lakewood, NJ, and a combination of collagenases B and D, Roche). However, the defined blendzyme mix reduced variation between preparations of collagenase, improving standardization between laboratories. Fifth, constant flow perfusion (3 ml/min) was used during the myocyte isolation rather than constant pressure. The constant pressure system (70 mm Hg) was satisfactory, but flow rates varied because coronary resistance typically increased at the onset of perfusion with digestion buffer. This increase in vascular resistance varied from heart to heart, and the consequent inconsistency in flow rates caused variations in the enzymatic digestion of the heart. Finally, calcium was reintroduced at room temperature, rather than 37 °C, which reduced spontaneous myocyte contracture. Calcium reintroduction was performed in a 60-mm sterile bacterial dish, which allowed visualization of the myocytes and helped reduce clumping that might occur if the myocytes were left in a tube.

Table 2. ***Adult mouse cardiac myocyte isolation***. Shown are myocyte isolation numbers for the Laboratory for the Development of Signaling Assays (LDSA) and the Cell Preparation and Analysis Laboratory (CPAL). The LDSA data show all preparations as single heart preparations, although, in many cases, myocytes were isolated from multiple hearts. The CPAL data show isolations of one heart or two hearts in which the myocytes were pooled. The values in the table for myocyte isolation are in millions (x 10^6) of cells per heart. Rod is the total number of rod-shaped myocytes; round is the total number of round myocytes; total is the sum of both; % rod-shaped is the number of rod-shaped myocytes/total myocytes. Data are Mean ± S.D.

	LDSA	CPAL	
Myocyte Isolation		1 heart	2 hearts
Number of preps	n = 152	n = 12	n = 52
Initial			
Rod	1.25 ± 0.14	1.49 ± 0.15	2.90 ± 0.48
Round	0.55 ± 0.11	0.53 ± 0.11	1.08 ± 0.24
Total	1.80 ± 0.19	2.02 ± 0.19	3.98 ± 0.57
% Rod-shaped	70 ± 4%	74 ± 4%	73 ± 5%
Prior to plating			
Rod	1.00 ± 0.10	1.27 ± 0.19	2.52 ± 0.52
Round	0.53 ± 0.08	0.46 ± 0.12	0.93 ± 0.26
Total	1.53 ± 0.14	1.73 ± 0.28	3.45 ± 0.69
% Rod-shaped	65 ± 4%	74 ± 4%	73 ± 5%

Culture of Adult Mouse Cardiac Myocytes

We next defined conditions to sustain myocytes in culture. Unfortunately, mouse myocytes, unlike rat and rabbit myocytes, are highly susceptible to contracture in culture, which causes rod-shaped myocytes to become rounded. While the cause of this contracture is unknown, it presents a significant obstacle to culturing adult mouse myocytes. Based on the methods of Zhou et al.(15), we developed a protocol for the culture of adult mouse cardiac myocytes that consistently maintains rod-shaped myocytes for 24 hours (Fig. 1). At 0 hours, myocytes were 90% rod-shaped. At 24 hours, cultured myocytes were 80% rod-shaped, and cultures maintained 88% of the viable, rod-shaped myocytes originally plated (Table 3). Therefore, combined with our ability to isolate myocytes from several hearts, sufficient numbers of myocytes could be cultured to perform large-scale signaling experiments.

We identified additional factors that improved the maintenance of viable, rod-shaped myocytes in culture (summarized in Table 1). The pH of the culture medium was the most critical factor. Myocytes were cultured in Modified Eagle's Medium (MEM) with Hanks' Balanced Salt Solution, supplemented with 0.1 mg/ml bovine serum albumin and penicillin 100 U/ml at 37 °C in a 2% CO_2 incubator. This resulted in medium with pH 6.9 to 7.0 (compared to normal medium with pH 7.4), similar to medium used by Zhou et al.(15), which provided a protective effect on the myocytes, possibly by reducing spontaneous contractile activity.

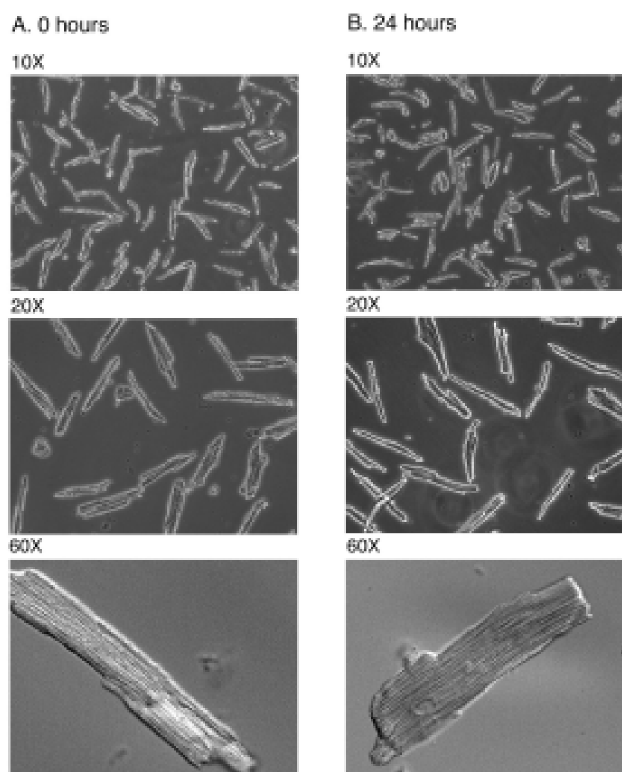

Fig. 1. **Adult mouse cardiac myocytes cultured for 24 hours.** Myocytes were photographed under both phase contrast (10X and 20X) and differential interference contrast (60X) microscopy after (**A**) 0 hr and (**B**) 24 hr in culture.

Cardiac Myocyte Signaling Responses

To assess the suitability of the adult mouse cardiac myocytes for signaling studies, we measured ligand-induced changes in the accumulation of cyclic adenosine monophosphate (cAMP) in the phosphorylation of a panel of signaling proteins and in excitation-contraction coupling. For all experiments, myocytes were cultured overnight (approximately 18 hours) before treatment with ligands.

Ligand induced changes in the accumulation of cAMP. To measure ligand-induced changes in cAMP, three sentinel ligands were used: (i) isoproterenol, a nonselective β-

Table 3. ***Adult mouse cardiac myocyte culture.*** Shown are myocyte culture data for the Laboratory for the Development of Signaling Assays (LDSA) and the Cell Preparation and Analysis Laboratory (CPAL). Myocytes are plated at 50,000 rod-shaped myocytes per 35-mm dish (55 per mm^2) in all experiments. 0 hr measurements are made after myocytes are plated and allowed to attach for 1 hr. % Rod-shaped is the number of rod-shaped myocytes/total myocytes. Plating efficiency is the number of rod-shaped myocytes attached after plating/number of rod-shaped myocytes plated (50,000 for a 35-mm dish). Data are Mean ± S.D.

	LDSA	CPAL
Myocyte Culture	n = 29	n = 59
0 hr		
% Rod-shaped	88 ± 8%	90 ± 3%
Rod-shaped MCs/35-mm dish	37944 ± 5979	40592 ± 5924
Total MCs/35-mm dish	43186 ± 7480	45125 ± 6625
Plating efficiency	76 ± 12%	81 ± 12%
24 hr		
% Rod-shaped	78 ± 6%	80 ± 6%
Rod-shaped MCs/35-mm dish	34090 ± 6450	35282 ± 7020
Total MCs/35-mm dish	44601 ± 9581	44162 ± 8253
% Rod-shaped vs. 0 hr	90 ± 10%	88 ± 15%

adrenergic receptor (β-AR) agonist that activates the Gs subtype of G proteins and adenylate cyclase; (ii) forskolin, a direct activator of adenylate cyclase; and (iii) carbachol, a muscarinic receptor agonist that activates the Gi subtype of G proteins and inhibits adenylate cyclase. Cyclic adenosine monophosphate concentrations were measured by enzyme-linked immunosorbent assay (ELISA)(PP00000015). Isoproterenol induced a rapid (30 seconds), seven-fold increase in cAMP that declined to a steady state plateau, about 60% of the peak, by three minutes (Fig. 2A). Forskolin, a direct activator of adenylate cyclase, caused a gradual increase in cAMP for the entire twenty-minute time course (Fig. 2B). Finally, carbachol inhibited increases in cAMP induced by either isoproterenol or forskolin (Fig. 2C and 2D). These observations are consistent with the known effects of these ligands on the accumulation of cAMP in cardiac myocytes from other species and whole heart.

Ligand-induced changes in the phosphorylation of signaling proteins. To measure ligand-induced changes in the phosphorylation of signaling proteins, five sentinel ligands were used: (i) phenylephrine (an α1-AR agonist) and (ii) angiotensin II, which are both hypertrophic agonists that activate Gq-coupled receptors; (iii) insulin and (iv) insulin-like growth factor 1, which are both hypertrophic agonists that activate the PI3-kinase signaling pathway; and (v) isoproterenol, a β-AR agonist. Protein phosphorylation was monitored by Western blot analysis using two phosphoprotein antibody mixtures (PP00000007). One antibody mixture contained phosphospecific antibodies to Akt (Ser 473), p38 MAP kinase (Thr 180/Tyr 182), and S6 ribosomal protein (Ser 235/236), and the second antibody mixture contained phosphospecific antibodies to ERK1/2 (Thr 202/Tyr 204), p70 S6 kinase (Thr 421/Ser 424), and phospholamban (Ser 16) (Fig. 3A). Phenylephrine significantly increased the phosphorylation of ERK and p38. Insulin and insulin-like growth factor-1 (IGF-1) rapidly increased the phosphorylation of Akt, p70 S6 kinase, and S6 ribosomal protein, all downstream targets of PI3-kinase. Isoproterenol greatly increased phosphorylation of phospholamban (about 40-fold) and also increased the phosphorylation of ERK, p38 MAP kinase, and S6 ribosomal protein (Fig. 3B-F). These results confirm previous reports for the ligand-induced changes in the phosphorylation of signaling proteins, for example, the increased phosphorylation of ERK in response to phenylephrine(18) and the increased phosphorylation of Akt with insulin in mouse heart in vivo(19).

Ligand-induced changes in myocyte excitation-contraction coupling (contraction and calcium transients). To assess excitation-contraction coupling in cultured adult mouse cardiac myocytes, we measured contractile responses and calcium transients in response to electrical stimulation and isoproterenol (supplemental method). Myocytes were cultured overnight and then loaded with the calcium indicator fura-2. Measurements of contraction (percent shortening) and calcium transients on individual myocytes were made with electrical stimulation (1 Hz, 20 V, 4 ms) before and after treatment with isoproterenol (Fig. 4A-D). Isoproterenol increased the extent of myocyte shortening (Fig. 4E; basal 3.79 ± 0.92 µm; isoproterenol 9.11 ± 0.59 µm; mean ± SEM; n = 7 for each; $P < 0.05$), the amplitude of the calcium transient (Fig. 4F; 171 ± 15%; mean ± SEM; n = 7; $P < 0.05$), and the rate of decline of the calcium transient (Fig. 4G; basal 0.185 ± 0.010 sec; isoproterenol 0.159 ± 0.004 sec; mean ± SEM; n = 7 for each; $P < 0.05$). The contractile and calcium responses to electrical stimulation in cultured adult mouse myocytes indicate intact excitation-contraction coupling mechanisms. In addition, increased myocyte shortening and calcium transients in response to isoproterenol

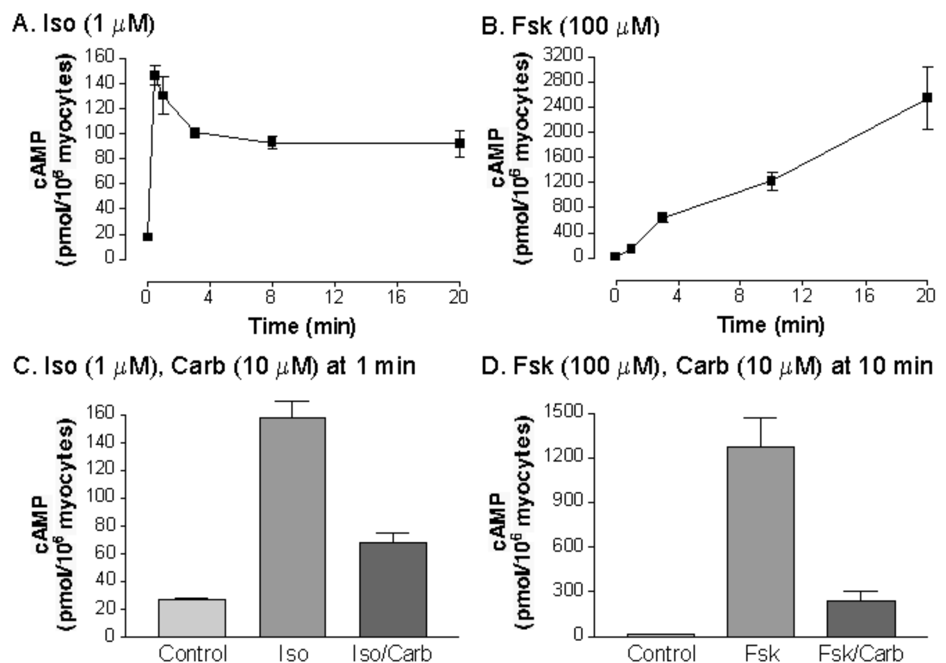

Fig. 2. **Accumulation of cAMP in myocytes treated with sentinel ligands.** (**A, B**) Myocytes were cultured for 18 hr (overnight) and then treated for 0, 1, 3, 8, or 20 min with (**A**) 1 µM isoproterenol or (**B**) 100 µM forskolin. After treatment, myocytes were lysed and the amounts of cAMP were determined by ELISA (PP00000015). (**C, D**) To assess inhibition of cAMP accumulation, myocytes were cultured overnight and then pretreated for 3 min with 10 µM carbachol and treated either (**C**) for 1 min with 1 µM isoproterenol or (**D**) for 10 min with 100 µM forskolin. After treatment, myocytes were lysed and the amounts of cAMP were determined by ELISA (PP00000015). Each graph shows mean ± S.E.M. n = 3, with each measurement made in duplicate.

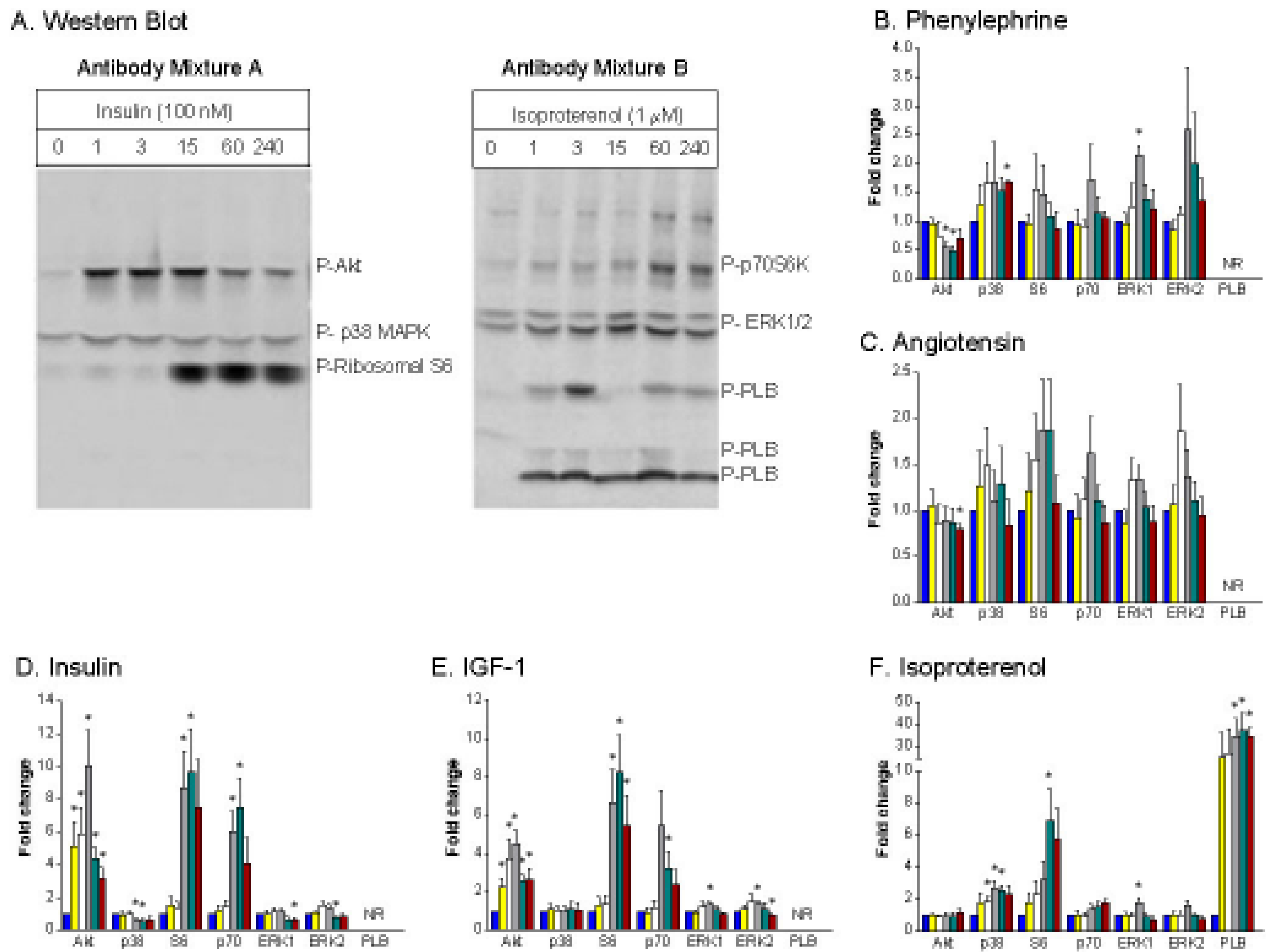

Fig. 3. **Phosphorylation of signaling proteins in myocytes treated with sentinel ligands.** Myocytes were cultured for 18 hr (overnight) and then treated for 0, 1, 3, 15, 60, or 240 min with five sentinel ligands: 30 μM phenylephrine (with 2 μM timolol, a β-adrenergic receptor antagonist); 100 nM angiotensin II; 100 nM insulin; 10 nM insulin-like growth factor-1; or 1 μM isoproterenol. After treatment, myocytes were lysed with SDS-PAGE sample buffer (PP00000132 and PS00000437) and the cell extracts were examined by Western immunoblotting (PP00000007) with two mixtures of phosphospecific antibodies. (**A**) Western blots were performed using two antibody mixtures. Antibody mixture A contained phosphospecific antibodies to Akt (Ser 473), p38 MAP kinase (p38 MAPK; Thr 180/Tyr 182), and S6 ribosomal protein (ribosomal S6; Ser 235/236), and antibody mixture B contained phosphospecific antibodies to p70 S6 kinase (p70S6K; Thr 421/ Ser 424), ERK 1 and 2 (ERK1/2; Thr 202/Tyr 204), and phospholamban (PLB; Ser16; pentamer, dimer, and single PLB bands are observed). The specific bands are identified to the right of each blot. The band densities for each phosphoprotein were measured (PP00000007), normalized to the band density for Rho-GDI, and the fold change was calculated relative to the 0 time point (= fold change defined as 1). (**B-F**) Each graph shows the fold change in phosphorylation for each of the five sentinel ligands at 0 (red), 1 (light blue), 3 (orange), 15 (green), 60 (purple), and 240 (blue) min, sequentially. For all bar graphs, data are mean ± S.E.M. n = 6 for three independent preparations from both the Cell Preparation and Analysis Laboratory and the Laboratory for the Development of Signaling Assays (LDSA), except for results with phenylephrine/timolol treated cells, n = 3, which were prepared in the LDSA. NR = no response; * $P < 0.05$ (t-test vs. 0 min).

correlated with the increase in phospholamban phosphorylation observed by Western blot analysis (8,15,20,21).

Long-term Culture of Adult Mouse Myocytes

A goal of the AfCS is to develop methods to interfere selectively with cell signaling proteins in cultured myocytes, using either vector-based RNA interference (RNAi) or the expression of dominant-negative signaling proteins. Using the AfCS protocol described herein, many of the cultured adult mouse myocytes did not maintain their rod-shaped morphology for longer than 24 hours*, which is not long enough to allow for the manipulation of gene expression. Therefore, we developed an alternative culture system that maintained viable, rod-shaped myocytes in culture for 72 hours.

*Although myocytes did not maintain their rod-shaped morphology past 24 hours, the myocytes were still viable as assessed by staining with vital dyes.

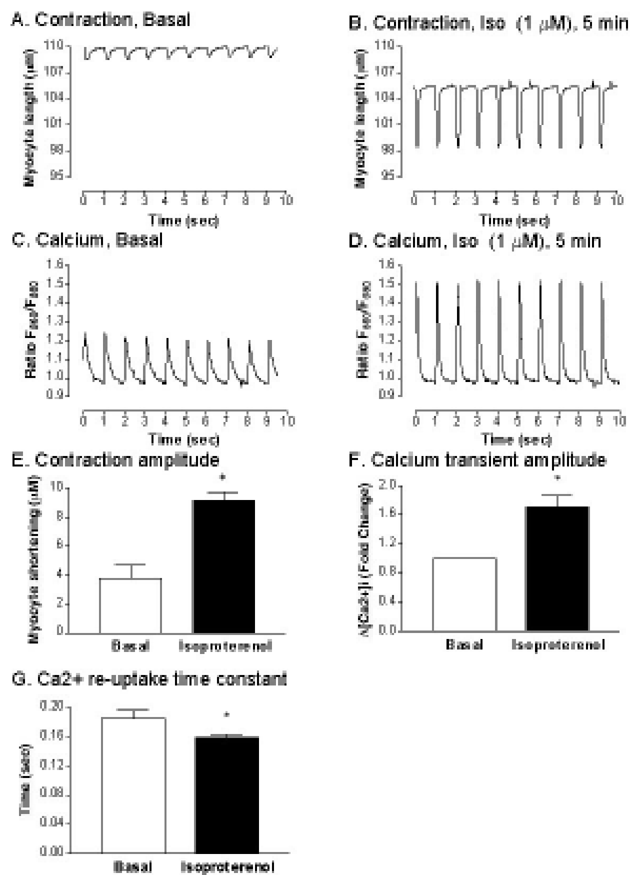

Fig. 4. **Calcium transients and contraction in myocytes treated with isoproterenol.** Myocytes were cultured for 18 hr (overnight) and then loaded with the calcium indicator fura-2AM at 25 °C in HEPES buffer. For all measurements, myocytes were electrically paced (1 Hz, with 20 V pulse amplitude and 4 ms pulse duration), and basal and ligand stimulated changes in length and calcium were recorded. (**A, B**) Contraction in a representative myocyte, measured as length change, is shown before and 5 min after treatment with 1 μM isoproterenol. The y-axis shows myocyte length in μm and the x-axis shows time in seconds. (**C, D**) Calcium transients are shown in a single myocyte before and 5 min after treatment with 1 μM isoproterenol. The y-axis shows fura-2 emission ratios (360 nm/380 nm) and the x-axis shows time in seconds. (**E**) Average myocyte shortening, (**F**) calcium transient amplitude (plotted as fold increase), and (**G**) calcium re-uptake time constant were measured before and 5 min after isoproterenol (1 μM). Data are mean ± SEM; n = 7 myocytes from two cultures; * $P < 0.05$.

Several agents were tested to improve the maintenance of viable, rod-shaped myocytes in culture (listed in Table 1). Viable, rod-shaped myocytes were best sustained in medium containing 2, 3-butanedione monoxime (BDM), a contractile inhibitor, as well as insulin, transferrin, and selenium (ITS). Myocytes cultured with 10 mM BDM and 10 μg/ml (1.7 μM) insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium showed no significant loss of rod-shaped morphology at 48 hours. At 72 hours, the number of rod-shaped myocytes was reduced by 30% (Fig. 5). Myocytes cultured without BDM and ITS showed a 20% loss of rod-shaped myocytes at 24 hours, but by 48 hours rod-shaped morphology was lost almost completely (Fig. 5). Similar losses of rod-shaped myocytes were observed with either BDM or ITS alone.

Because the insulin concentration used (0.17 μM) activates insulin-dependent signaling pathways, we tested lower concentrations of ITS. A 1000-fold dilution of the ITS supplement (10 ng/ml, 1.7 nM, insulin, 5.5 ng/ml transferrin, 5 pg/ml selenium) maintained myocyte rod-shaped morphology almost as well. Compared to myocytes at 0 hours, 99% of myocytes were rod-shaped at 24 hours in medium with the reduced ITS concentration, 83% at 48 hours, and 66% at 72 hours.

The preservation of myocyte morphology by BDM might involve one or more previously proposed mechanisms, including the regulation of calcium entry through the L-type calcium channel(22) and Na^+/Ca^{2+} exchanger(23), the regulation of calcium content in the sarcoplasmic reticulum(24), and the inhibition of contraction through dephosphorylation of troponin I and phospholamban(25). Regardless of the mechanism, at the concentration used, BDM appeared to have a minimal effect on cultured myocytes. It was previously shown that contraction is not inhibited by the presence of BDM in isolated myocytes(24). Furthermore, preliminary experiments suggested that ligand-induced changes in cAMP or phosphoprotein phosphorylation were not altered qualitatively by the presence of BDM in the culture medium. The effect of the ITS medium supplement to maintain rod-shaped myocyte morphology was most likely related to the known metabolic effects of insulin to stimulate glucose and amino acid uptake. However, at higher concentrations, insulin might also inhibit apoptosis by stimulation of the PI3-kinase signaling pathway. Therefore, the long-term culture of myocytes in medium supplemented with BDM and ITS appears to provide a stable platform for signaling experiments, and additional experiments are underway to characterize further myocytes in long-term culture.

Adenoviral Infection of Adult Mouse Myocytes

An important requirement for developing methods to investigate cell signaling is a system for introducing exogenous DNA to myocytes. Adenoviral infection has been used for high-efficiency gene transduction in both rat and mouse cultured myocytes (> 90% efficiency)(15,26,27). To test the capacity for adenoviral-mediated gene transduction in long-term cultures of adult mouse cardiac myocytes, myocytes were infected with an adenovirus-β-galactosidase (β-gal) reporter gene (supplemental method). Low viral titers showed variable expression and a lower percentage of infected cells at 24 hours (Fig. 6A). Both the level of β-gal expression and the percentage of infected cells increased with increased viral titer (multiplicity of infection [MOI] 30, 39% blue myocytes; MOI 100, 74%; MOI 300, 90%; MOI 1000, 96%). After 72 hours (Fig. 6B), β-gal expression was extensive even at the lowest titer measured (MOI 30, 96% blue myocytes). Myocyte survival was decreased at the highest

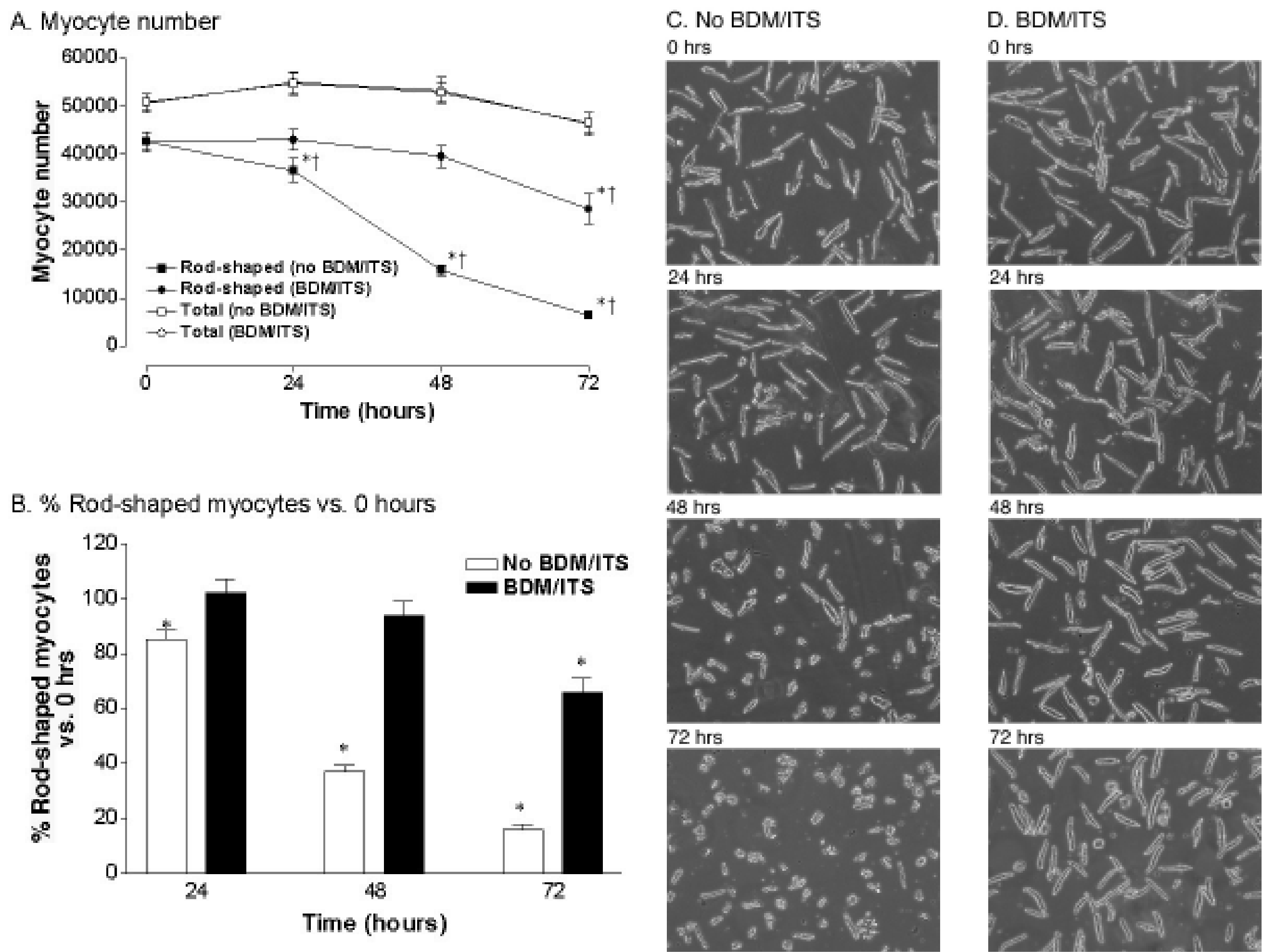

Fig. 5. **Myocytes cultured with 2, 3-butanedione monoxime (BDM) and insulin, transferrin, and selenium (ITS) for 72 hours.** (**A**) Myocytes were plated at a density of 50,000 rod-shaped myocytes per 35mm culture dish and were cultured for 72 hr in medium with or without 10 mM BDM, 10 µg/ml insulin, 5.5 µg/ml transferrin, and 5 ng/ml selenium. Myocytes (both rod-shaped and round myocytes) were counted at 0, 24, 48, and 72 hr. Data show total myocyte number (rod + round, closed symbols) and rod-shaped myocyte number (open symbols) in culture medium alone (squares) or culture medium with BDM and ITS (circles). Data are mean ± S.E.M. for n = 11; * $P < 0.05$ for myocytes in medium alone vs. myocytes with 10 mM BDM and ITS at each time point; † $P < 0.05$ for myocytes at each time point vs. 0 hr for each group. (**B**) The percentage of rod-shaped myocytes at 24, 48, or 72 hr compared to 0 hr calculated by the formula: (number of rod shaped myocytes at X hrs)/(number of rod shaped myocytes at 0 hrs). Data are mean ± S.E.M. for n = 11; * $P < 0.05$ vs. 0 hr for each group. Myocytes cultured in (**C**) medium alone or (**D**) medium supplemented with BDM and ITS were photographed under phase contrast microscopy (10X) after 0, 24, 48, and 72 hr in culture.

titer (MOI 1000, 51% survival), possibly due to adenovirus-induced toxicity. The effective expression of this reporter gene for 72 hours suggests that the expression of dominant-negative signaling proteins or vector-based RNAi for selective protein knock-downs should be possible in cultured adult mouse myocytes.

In summary, we present a protocol for reproducibly isolating large numbers of viable, rod-shaped myocytes and maintaining them in short-term culture (24 hours). Under these conditions, both ligand-induced signaling responses and excitation-contraction coupling remain intact. Further, we present a long-term culture system (72 hours), using medium supplemented with BDM and ITS to maintain rod-shaped myocytes for extended duration in culture. Under these conditions, 95% to 100% of cultured myocytes can be transduced with adenovirus to express exogenous DNA for 72 hours. Therefore, this protocol presents the opportunity for developing methods to interfere with cell signaling.

Methods and Protocols

Detailed methods and protocols used for these studies are available in PDF format and accessible via the online version of this report.

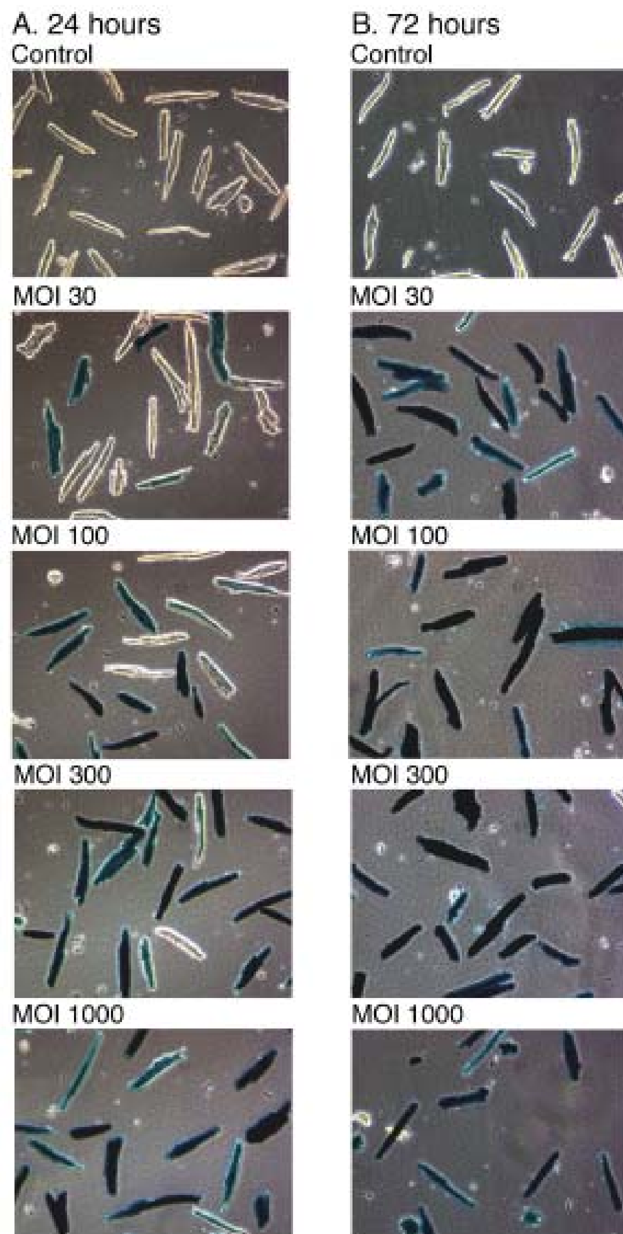

Fig. 6. **Adenovirus infection and β-galactosidase expression in cardiac myocytes.** Myocytes were cultured for 72 hr in medium supplemented with 10 mM BDM, 10 µg/ml insulin, 5.5 µg/ml transferrin, and 5 ng/ml selenium. Myocytes were infected at 0 hr with increasing titers (MOI 30 to 1000) of adenovirus containing a β-galactosidase transgene (β-gal). β-gal activity was assayed at (**A**) 24 and (**B**) 72 hr and myocytes were photographed under phase contrast microscopy (10X).

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