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Cell Calcium xxx (2007) xxx–xxx

cell calcium

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A primary culture system for sustained expression of a calcium sensor in preserved adult rat ventricular myocytes

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Received 6 February 2007; received in revised form 27 March 2007

Abstract

For studying heart pathologies on the cellular level, cultured adult cardiac myocytes represent an important approach. We aimed to explore a novel adult rat ventricular myocyte culture system with minimised dedifferentiation allowing extended experimental manipulation of the cells such as expression of exogenous proteins. Various culture conditions were investigated including medium supplement, substrate coating and electrical pacing for one week. Adult myocytes were probed for (i) viability, (ii) morphology, (iii) frequency dependence of contractions, (iv) Ca^{2+} transients, and (v) their tolerance towards adenovirus-mediated expression of the Ca^{2+} sensor "inverse pericam". Conventionally, in either serum supplemented or serum-free medium, myocytes dedifferentiated into flat cells within 3 days or cell physiology and morphology were impaired, respectively. In contrast, myocytes cultured in medium supplemented with an insulin–transferrin–selenite mixture on substrates coated with extracellular matrix proteins showed an increased cell attachment and a conserved cross-striation. Moreover, these myocytes displayed optimised preservation of their contractile behaviour and Ca^{2+} signalling even under conditions of continuous electrical pacing. Sustained expression of inverse pericam did not alter myocyte function and allowed long lasting high speed Ca^{2+} imaging of electrically driven adult myocytes. Our single-cell model thus provides a new advance for high-content screening of these highly specialised cells.

Keywords: Calcium; Cardiac; Culture; Adult ventricular myocyte; Imaging; Contraction; Rat

1. Introduction

The acute isolation of adult cardiac myocytes has been established decades ago [1] to investigate the cells' physiological behaviour. In contrast, studies requiring extended culture periods, e.g. for protein expression or knock-down, have always been limited to a couple of days in culture due to extensive morphological and physiological alterations of the adult myocytes occurring shortly after isolation [2]. This restriction could not be compensated for adequately by the creation of cardiac cell lines since they do not represent cardiac myocyte physiology well enough [3]. Currently, neonatal myocytes serve as a limited model for the adult cell, but it has to be noted, that in comparison to adult myocytes neonatal cells display a different phenotype and genotype. Nevertheless, long-term culturing of these cells even in larger quantities is routine.

In conventional culture, isolated adult rat cardiomyocytes rapidly change from a "brick-like" structure towards a more stellated, neonatal-like shape. Moreover, their size increases considerably [4]. In serum-free culture medium, adult cardiac myocytes from guinea-pigs, rats, rabbits and mice are usually quiescent and retain their viability and unique rod-shaped morphology for at least a couple of days [5–7]. These cells maintain highly organised membrane and myofibrillar structures that support contractions induced by electrical stimulation. Thus, they appear suitable to short-term (1–3 days) virus-mediated expression of exogenous proteins [8]. For future studies requiring long-term expression of exogenous proteins or vector-based RNA interference (RNAi) to

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doi:10.1016/j.ceca.2007.04.001

knock-down protein expression it appears essential to employ longer culture periods without a loss of morphology and physiology of the freshly isolated cells. Moreover, experimental manoeuvres inducing "slow-onset" cellular responses will also entail long-term culturing of the myocytes. Additionally, molecular biology techniques such as Western blotting demand large amounts of proteins from homogeneous cell populations. Thus, culturing set-ups are needed that offer the possibility to electrically stimulate large homogeneous populations of cells simultaneously. A decade ago, an adult rat ventricular myocytes culture system was developed with conditions that allow short-term (3 days) culture together with the ability to impose arbitrary electrical pulse protocols [9].

The goal of the present study was to use that basic approach and refine it to a long-term culture system (1 week) with diminished cellular dedifferentiation. We tested the suitability of our system in multiple ways including morphology, survival rate, contractile behaviour, Ca^{2+} signalling and success for adenoviral mediated expression of an exogenous protein (inverse pericam, a fluorescence calcium indicator based on calmodulin [10]).

2. Methods

2.1. Isolation and primary culture of adult rat ventricular myocytes

We adopted a protocol for cell isolation based on established procedures in rabbit and mouse [11,12] for the rat heart. Adult male Wistar rats (6–12 weeks old, 200–400 g) were handled and sacrificed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were anaesthetised by an intraperitoneal injection (i.p.) of pentobarbital sodium, 160 mg/kg body weight (Narcoren; Merial, Germany). Directly afterwards, we injected (i.p.) 0.5–1 ml (according to the body weight) of a citrate (40 mM) solution to prevent formation of blood clots. Ten minutes later, the animal was killed by decapitation. The heart was flushed with 10 ml of ice-cold Ca^{2+}-free solution (CFS) containing (in mM): NaCl 134, Glucose 11, KCl 4, $MgSO_4$ 1.2, Na_2HPO_4 1.2, HEPES 10 (Merck, Germany) (pH adjusted to 7.35 with NaOH). After that, the heart was removed, attached to a Langendorff apparatus and perfused retrogradly with O_2 saturated CFS containing 200 μM EGTA at a rate of 4 ml/min for 5 min. The perfusate was then changed to O_2 saturated CFS containing collagenase type I (Worthington, New Jersey, USA) at a final concentration of 1 mg/ml for 25 min.

The ventricles were removed, minced and placed in O_2 saturated CFS containing 1 mg/ml collagenase (at 37 °C in a water bath for 2 min). After sedimentation, the resulting supernatant was discarded and the pellet was mixed and resuspended in 20–25 ml of O_2 saturated CFS and incubated as above. The supernatant was discarded again and the pellet was mixed and resuspended in 20–25 ml of O_2 saturated low-Ca^{2+} solution (LCS) containing 50% of CFS and 50% of high-Ca^{2+} solution (HCS) and incubated as above. HCS is composed of CFS supplemented with 0.09% of DNAse and 200 μM of Ca^{2+}. Furthermore, the supernatant was discarded, the pellet was resuspended in 20–25 ml of O_2 saturated HCS and incubated as above. Then rat ventricular myocytes were released from the soft tissue by gentle trituration. The cell suspension was plated into "peel-off" culture flasks (Techno Plastic Products AG, Switzerland), the internal bottom surface of which were coated with poly-L-lysine (500 μg/ml; Sigma, USA) or with a mixture of extracellular matrix proteins (ECM, 1.11 mg/ml; Harbor Bio-Products, Norwood, MA, USA). The myocytes were allowed to settle down for approximately 1 h in medium M199 with Earle's modified salts, glutamine (Biowest; Nuaille, France), 100 μg/ml Penicillin/Streptomycin and 50 μg/ml Kanamycin (PAA Laboratories, Austria). In addition to the control condition (pure medium), the medium was supplemented with either 5% fetal calf serum (FCS supplemented medium) or 870 nM insulin, 65 nM transferrin and 29 nM Na–selenite (Sigma, USA) (ITS supplemented medium). Myocytes were cultured in an incubator at 37 °C with a 5% CO_2 atmosphere. After plating the medium was changed at 1 h, day *in vitro* (DIV) 1, 3 and 6 with warm fresh medium, in order to remove the loosely attached cells.

For the experiments involving the viral gene transfer the cells were plated on ECM-coated cover slips, placed in 12-well plates and kept in M199 medium supplemented with ITS. Adenovirus-mediated gene transfer was initiated 1 h after cell plating to allow a fast protein expression. The myocytes were transfected with a multiplicity of infection (MOI) of 5–20 plaque-forming units/cells. The regime for exchanging the culture medium was as described above.

The continuous electrical stimulation was performed at 37 °C. All other experiments were carried out at room temperature (20–22 °C).

2.2. Electrical stimulation

For electrical stimulation of entire cell populations we designed and built Plexiglas lids, resistant to heat sterilisation, as shown in Fig. 1A with the following features: (i) two parallel carbon electrodes for electrical field stimulation; (ii) standardised connectors for external electrical pulses; and (iii) silicone sealing for taking the culture flask out of the incubator while maintaining sterile internal conditions. The set-up for electrical stimulation comprised a custom-made high-current pulse amplifier (cp. Fig. 1B; Babraham Tech^{nix}, Cambridge, UK). The software "Cardiac Stimulator" was running under LabView software allowing continuous pacing of culture flasks at an adjustable frequency, cp. Fig. 1C. We used 0.2 Hz throughout all culture conditions involving pacing of cardiac myocytes.

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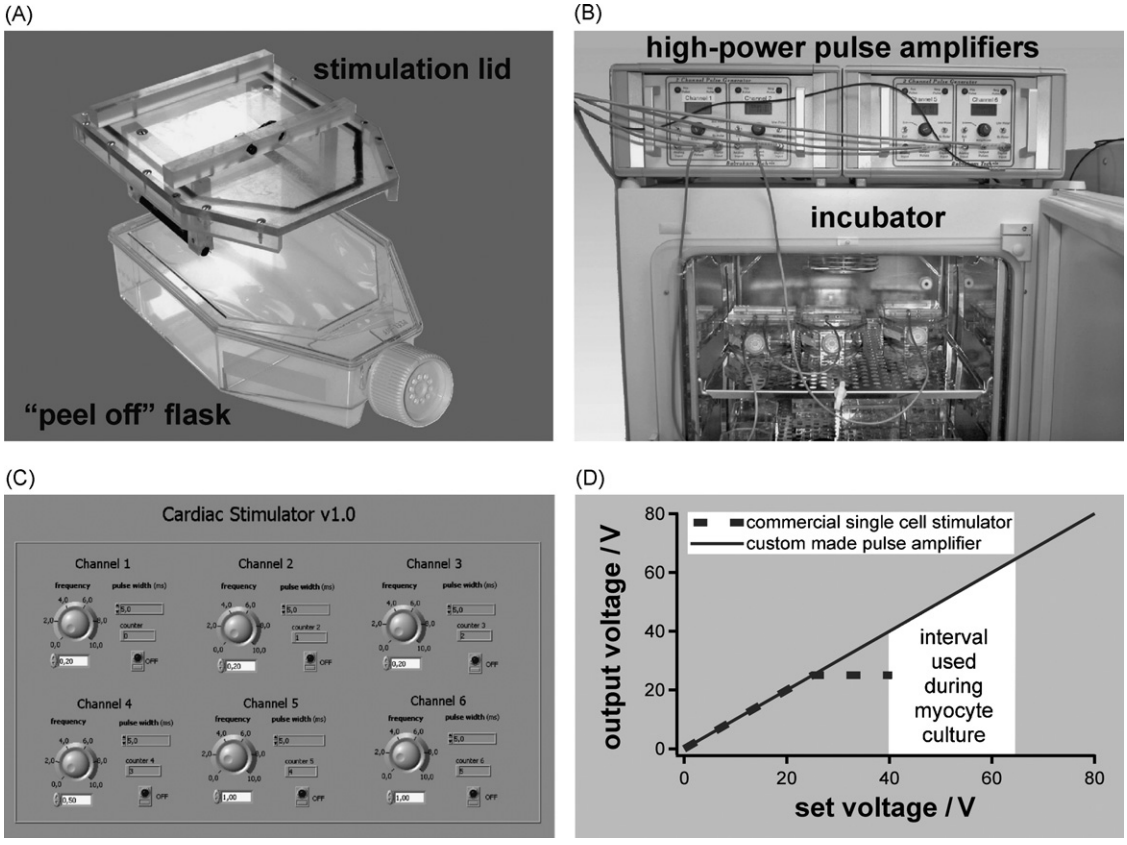

Fig. 1. Electrical stimulation and prolonged culture of adult cardiomyocytes. Panels (A–C) illustrate components of the optimised culturing system. (A) Shows the custom-made stimulation lid (top) and "peel-off" flask (bottom), (B) two high-power amplifiers on top of a standard cell incubator connected to several "peel-off" flasks for electrical stimulation of myocyte populations. Panel (C) depicts the graphical user interface of the LabView based software "Cardiac stimulator" controlling the electrical pacing of the myocytes in the flasks. Panel (D) displays the relationship of set voltage (*x*-axis) and output voltage (*y*-axis) of a commercial single cell stimulator (dahsed line) and the custom made pulse amplifier (solid line) connected to an individual culture flask filled with medium as shown in panels (A) and (B). The typically required voltages used during myocyte culture are highlighted.

2.3. Measurements of cell length change

Electrical stimulation induced cell-length changes were recorded with a fast video camera (sampling rate 240 Hz) from cells maintained in the culture flask. For this we transferred the flasks from the incubator onto the stage of an inverted microscope (Eclipse TS100, NIKON, Japan) equipped with a cell-length measurement system (IonOptix Corporation, USA). The system directly put out cell-length changes that were further analysed in Igor Pro software (Wavemetrics, USA) with custom-made macros.

2.4. Fluorimetric Ca^{2+} recordings

Global Ca^{2+} transient were measured with either fura-2 or the Ca^{2+} sensitive fluorescent protein inverse pericam [10]. To perform such recordings, cardiac myocytes were seeded on coated glass cover slips that were placed into culture flasks (for fura-2) or into wells of a 12-well plate (for inverse pericam) before seeding. For fluorescence recordings the cover slips were mounted in a custom made chamber on the stage of an inverted microscope (TE2000U, NIKON; Japan) attached to video-imaging hardware. Imaging was carried out through a 20× oil-immersion objective (Planfluor 0.75 NA, NIKON, Japan). The system comprised a video camera (for fura-2: Imago, TILL Photonics, Germany; for inverse pericam: iXon DV887, Andor Inc., Ireland) and a monochromator for excitation (Polychrome IV, TILL Photonics, Germany).

For the fura-2 recordings cover slips were loaded with dye (fura-2-AM, 0.4–0.75 μM, from a stock of 1 mM in DMSO/20% pluronic) for 30 min at room temperature. Prior to recording, the loading solution was exchanged with extracellular solution (ES) composed of (in mM): NaCl 135, KCl 5.4, $MgCl_2$ 1, glucose 10, $CaCl_2$ 2, HEPES 10 adjusted to pH 7.35 with NaOH. Imaging was performed by exciting the cells at the Ca^{2+}-dependent wavelength (380 nm) and recording the fluorescence signal (>440 nm; image exposure duration: 15 ms). The excitation at 380 nm was interrupted every 50th image by recording a single image at the Ca^{2+}-independent, isosbestic excitation wavelength of 355 nm (see Fig. 4A). For calculating ratiometric data we linearly interpolated between the 355 nm-images and ratioed the fluorescence values against the corresponding 380 nm-image to obtain true F_{355}/F_{380}-fura-2 ratio data at an acquisition frequency of

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66 Hz. This ratioing and further semi-automatic peak detection was performed in Igor Pro software running custom made macros after averaging the fluorescence of regions of interest in the imaging software.

Inverse pericam is a chimeric protein comprising a circularly permuted green fluorescent protein and calmodulin [10]. Imaging of the inverse pericam fluorescence was performed by exciting the fluorophore at 490 nm and recording the fluorescence through a 510 nm long-pass filter (image exposure duration 15–20 ms, resulting in an imaging frequency of 50–66 Hz). Single fluorescence images were obtained by exporting entire movies as multi-page TIFF files and processing them in ImageJ (W. Rasband, NIH, USA). For self ratio traces we calculated the $F_o/\Delta F$ ratio since the emitted fluorescence of the inverse pericam decreased with increasing Ca^{2+} concentrations, thus the term "inverse".

2.5. Adenovirus construction

Generation of recombinant adenoviruses was accomplished using the Transpose-Ad™ Adenoviral Vector System (MP Biomedicals, USA) according to the manufacturer's instructions. A pCR259 adenovirus transfer vector encoding for the calcium-sensitive fluorescence protein inverse pericam was transformed in HighQ-1 Transpose-Ad™ 294 competent cells, a bacterial cell line carrying the Transpose-Ad™ 294 plasmid and a plasmid encoding a trans-acting Tn7 transposase. After a Tn7-based transposition, recombinant adenoviral genome was purified from bacteria and transfected into the QBI-HEK 293 cell line using Lipofectamine 2000 (Invitrogen, Germany). In this cell line, the recombinant adenoviruses were generated and propagated.

The pcDNA3-inverse pericam vector was kindly provided by Dr. Atsushi Miyawaki (Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan).

2.6. Data analysis

Results were analysed using a Mann–Whitney rank sum test (SigmaStat software, USA). Effects were regarded as significant when $p < 0.05$ (marked with an asterisk). The results are expressed as mean values ± S.E.M.

3. Results

3.1. Electrical field stimulation of cardiac myocytes

In our peel off flask/lid system (see Fig. 1A and Section 2.2) a field voltage of 40–65 V at pulse durations of 5 ms (rectangular pulses) was necessary to trigger a visible contraction in at least 75% of the isolated myocytes. In order to generate these pulses, commercially available pulse generators such as the MyoPacer (IonOptix Corp., USA) were not sufficient, because (i) their voltage output is limited to 40 V and (ii) the electric current necessary for the peel off flask/lid system was higher than the limit of the total output power of the MyoPacer (compare Fig. 1D). This restricted the highest achievable output voltage to 25 V (measured with two independent MyoPacers). It has to be mentioned here that such amplifiers had been designed solely for single cell experiments and our findings might simply indicate design specific limitation. We thus obtained custom made high power and fast switching pulse amplifiers (cp. Fig. 1B and Section 2.2), which delivered enough power to simultaneously drive four of our peel of flasks (per output channel) at a maximal voltage of more than 80 V (voltage stability confirmed; voltage change during the pulse <5%). For this the hardware generated an electrical current of approximately 2.1 A (calculation based on a specific electrical resistance of culture medium of approximately 125 Ω cm, an electrode distance of 8 cm and a cylindrical electrode geometry of a length of 7.3 cm and a diameter of 6 mm).

The comparison between a single cell stimulator and our custom made pulse amplifier is depicted in Fig. 1D. For this we connected a single culture flask filled with medium to the amplifier and measured the actual output voltage for a range of set voltages.

During the myocyte culture the voltage was re-adjusted on a daily basis. With this we ensured to always drive at least 75% of the muscle cells which was inspected visually through a microscope. We observed that the voltage necessary for that increased from about 40 V at DIV0 to approximately 65 V at DIV6. In our studies we applied the pulses (square-shaped; 5 ms in duration) at a constant frequency of 0.2 Hz for the entire culture period.

3.2. Long-term culture of cardiomyocytes: morphology and survival rates

Isolation of adult rat ventricular myocytes yielded more than 80% living cells of which more than 70% displayed a rod-shaped morphology (data not shown). Initially, we evaluated various culture conditions based on the light microscopic morphology of the myocytes during a week of culture. Fig. 2A summarises our findings for five different culture conditions. Each row of panels depicts the culture conditions while the columns represent successive DIVs.

The first two rows show typical results when culturing adult rat ventricular myocytes in a supplement-free medium (Fig. 2A). Over the time course of 7 days, less than 50% of the cells were able to largely retain their elongated phenotype. We found that after a few days the myocytes developed numerous small vesicles or vacuoles as indicated by the two insets in the first row of images, the development of which were independent of the substrate coating (Fig. 2A 1st and 2nd rows).

When cultured in medium supplemented with 5% FCS, the myocytes rapidly "de-differentiated" in their morphology (Fig. 2A). This process was so fast that from DIV3 onwards, reliable contraction measurements based on edge detection (c.p. 2.3) were difficult due to massive changes in

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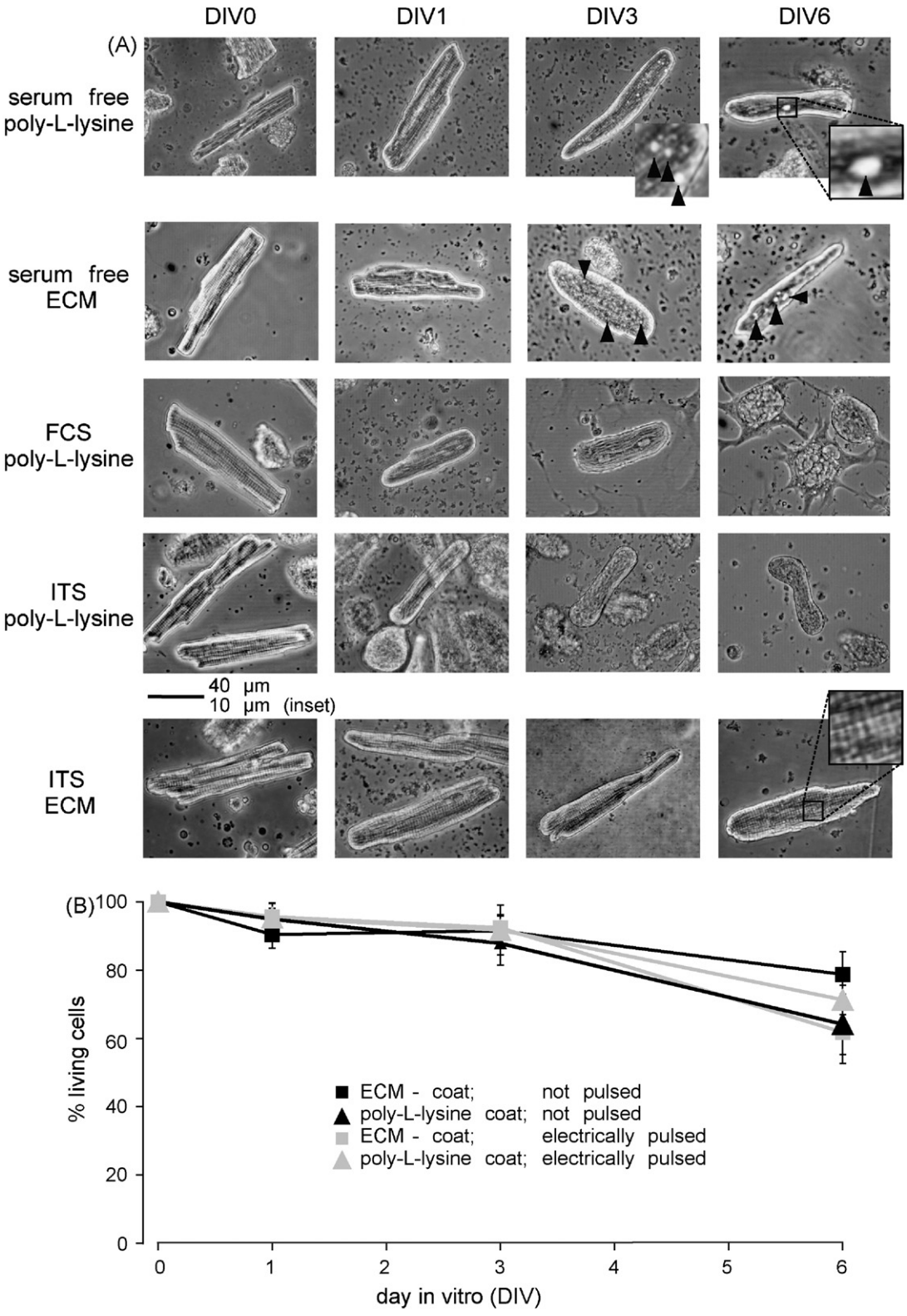

Fig. 2. Morphological properties and survival rates of cardiomyocytes in long-term culture. Panel (A) depicts transmission images of typical adult rat ventricular myocytes under various culturing conditions. The rows represent different culture conditions for the time points shown (columns). For selected combinations part of the cell body has been redrawn in a magnified inset (the same reference length bar indicates 40 μm for the images and 10 μm for the magnified insets). The black arrowheads in the panels of the first two rows highlight the occurrence of numerous vesicles and vacuoles. In panel (B), we have plotted the normalised yield of living cells (as percentage of the total number of cells, i.e. living and dead) versus the time of culturing. The data were taken from 4 to 8 different rat heart preparations for the two most promising culture conditions. Rectangular symbols indicate ITS supplemented culture conditions while triangles refer to media without serum.

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the cell geometry, i.e. the majority of cells were rounded up (>65%). Furthermore, at DIV6 most cells started to develop lamellipodia-like structures and adopted a flattened "fried egg" shape (Fig. 2A, third row, rightmost image). In comparison to serum conditions, for myocytes cultured under serum-free and ITS-supplemented conditions (Fig. 2A, two lower rows) such morphological "de-differentiation" was significantly reduced regardless of the substrate coating (poly-L-lysine: Fig. 2A 4th row or ECM-coated substrates: Fig. 2A bottom row). Even after 6 days in culture >32% elongated myocytes were present with the poly-L-lysine coating, without any lamellipodia-like structures. The rate of elongated cells on the flask surfaces coated with ECM was even exceeding those rates (>42%).

From these results we concluded that the two most favourable culture conditions so far were either without any medium supplements on poly-L-lysine coating or with ITS-supplement on ECM-coated substrates. We thus investigated those two conditions further to identify the superior one with respect to cell survival and conservation of the morphology.

Fig. 2B compares the survival rates of the myocytes under the two most promising culture conditions, i.e. ITS/ECM and no serum/poly-L-lysine for pulsed and non-pulsed cells. From these data it became apparent that the overall survival rates of ITS/ECM cultured cells were not significantly different compared to the non supplemented culture conditions, a finding observed for pulsed and non-pulsed conditions. This obviously indicated that electrical pacing did not exert a detrimental effect on cell survival.

Interestingly a higher total number of cells was found on the ECM-coated surfaces after the isolation, plating and initial washing steps (data not shown) in comparison to the poly-L-lysine substrate coating. This might indicate a stronger interaction between the cells and the coating when seeded onto ECM-coatings. Moreover, we found that reliable cell length measurements with poly-L-lysine were difficult during the first 4 h after seeding, because a large proportion of the plated cells displayed highly increased spontaneous activity that ceased over the time course of a few hours after plating (data not shown).

When we visually inspected the myocytes under both culturing conditions at DIV6 we found that in comparison to the ITS/ECM condition the cells in serum free medium displayed (i) numerous vesicles and/or vacuoles (see DIV6, first row in Fig. 2A) and (ii) a loss of apparent cross-striation (compare DIV6 first and last row in Fig. 2A).

3.3. Long-term culture of cardiomyocytes: analysis of cross-striation

In order to quantify the presence of cross-striation as an indication for the conservation of highly organised contractile filaments and structures we calculated spatial power spectra from elongated adult rat cardiac myocytes under serum free and ITS/ECM conditions (Fig. 3). For this, we generated intensity profiles (Fig. 3Aa and b, black lines) along the longitudinal axis of the myocytes at DIV0 and 6 and constructed power spectra (Fig. 3B). For cells at DIV0 we consistently found a peak at the spatial frequency of $0.56 \pm 0.015\ \mu m^{-1}$ ($n = 6$ cells, translating to a regular structure with a repetition every 1.78 μm) regardless of the particular culture condition. This value for the spatial frequency was very close to the one expected for sarcomeric structures (i.e. 1.8 μm sarcomeric length; [13]). From this we concluded that the regular banding visually identified in the myocytes at DIV0 was indeed caused by the typical cross-striation generated by the regular arrangement of the contractile filaments and t-tubules. A similar analysis was performed with cells at DIV6 either in ITS/ECM or in serum-free/poly-L-lysine conditions. We found that the cells in the former condition displayed a frequency peak that appeared slightly shifted towards higher frequencies ($0.61 \pm 0.027\ \mu m^{-1}$ for

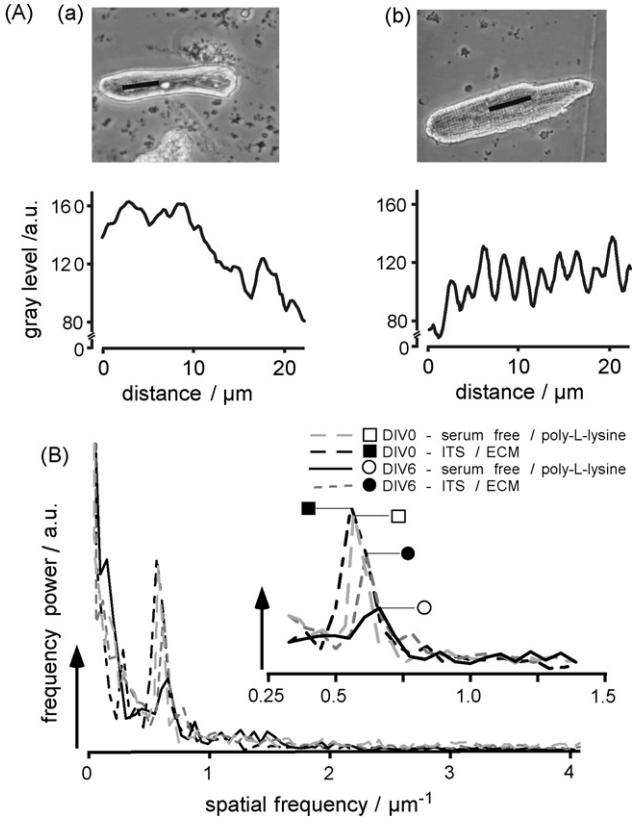

Fig. 3. Cross-striation of cardiomyocytes in long-term culture. Panels (A–B) compare rat ventricular myocytes at DIV0 and DIV6 under various culturing conditions. In order to quantify cross striation we recorded intensity profiles (panel A) of myocyte images taken with a regular phase-contrast transmission microscope along their longitudinal axis. The left image depicts a myocyte at DIV6 cultured in serum free medium on poly-L-lysine (Aa) whilst the right image represents a myocyte at DIV6 cultured in ITS supplemented medium on ECM (Ab). For (B) we performed a powerspectral analysis of such line profiles taken from myocytes at DIV 0 and DIV 6. The inset illustrates a magnified view onto the power peak around spatial frequencies of $0.56\ \mu m^{-1}$. Symbols highlight the particular peak. Details for the construction of the powerspectra can be found in the Section 2. These results were typical for all cells analysed ($n = 9$ at DIV0 and $n = 6$ for each DIV6 condition; cells were taken from three rat hearts).

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DIV6 versus $0.56 \pm 0.018\ \mu m^{-1}$ for DIV0, $n = 6$ for each DIV, translating into 1.64 μm for DIV6 versus 1.78 μm for DIV0). The amplitude in that peak was also reduced to 68.9% ± 10% ($n = 6$). Even for the cells cultured in serum free/poly-L-lysine conditions at DIV6 we could identify a spectral frequency peak in the very same region, although as described above cross-striation was often absent when analysed by visual inspection only. Nevertheless, these peaks were shifted towards higher spatial frequencies even further ($0.66 \pm 0.03\ \mu m^{-1}$, $n = 5$ cells, translating into 1.5 μm). In addition the amplitude in that peaks was significantly reduced to 24.5 ± 15% ($n = 5$) when compared to the DIV0 condition (see Fig. 3B, inset; compare open circle with other symbols).

After this initial analysis of the culture conditions we set out to comprehensively investigate the physiology of the cultured cells. For this we analysed the frequency dependence of their contractility and Ca^{2+} transients during electrical pacing from DIV0 to DIV6 under various culture conditions.

3.4. Shortening-frequency relationship

Fig. 4A exemplifies the stimulation protocol used for the cell length and for the Ca^{2+} measurements described below. Fig. 4B depicts the time course of cell length changes (0.5 Hz, pulse duration 5 ms) at DIV0. While the first contraction was strong, a typical progressive decay in the contraction amplitude could be observed, a phenomenon termed post-rest potentiation [13]. In Fig. 4C and D traces are exemplified for two different culture conditions (left: no medium supplement on poly-L-lysine; right: ITS supplemented medium on ECM coating) at DIV1 (Fig. 4C) and DIV6 (Fig. 4D). While at DIV1 both cells displayed post-rest potentiation, the myocyte cultured without supplement showed a greatly diminished potentiation at DIV6 while the cell with ITS/ECM still revealed post-rest potentiation.

It is noteworthy that the absolute maximal cell length changes decreased over time from DIV3 onwards in all conditions tested (data not shown). Most likely this reduction of the absolute amplitudes of cell shortening is attributed to an increase of the interaction between cells and substrates (see details in Section 4). Because of this we analysed changes of the post-rest behaviour of contraction (normalised to the pre-stimulation contractions) rather than absolute twitch amplitudes.

In order to quantify the degree of post-rest potentiation we calculated the relative change of contractility by ratioing the twitch amplitude under steady-state conditions (mean value of the last five peaks; T_{ss}) by the initial contraction amplitude (T_1) as depicted in Fig. 4A. Fig. 5 summarises the frequency dependence of that ratio and its relation to the culture conditions. We found that under serum free/poly-L-lysine, FCS/poly-L-lysine and ITS/poly-L-lysine conditions, the negative frequency dependence of the T_{ss}/T_1 ratio was lost between DIV3 and DIV6. In contrast, myocytes cultured in ITS-supplemented medium on ECM coated substrates largely retained the negative frequency dependence (Figs. 5Ad, Bd, Cd, Dd for DIV6 data). We observed a particular dramatic change for cells cultured in FCS-supplemented medium. The negative frequency dependence turned into a positive relationship termed post-rest decay (Fig. 5Bd, closed symbols).

When we compared data from cells not stimulated during the culture period with those derived from pulsed cell pop-

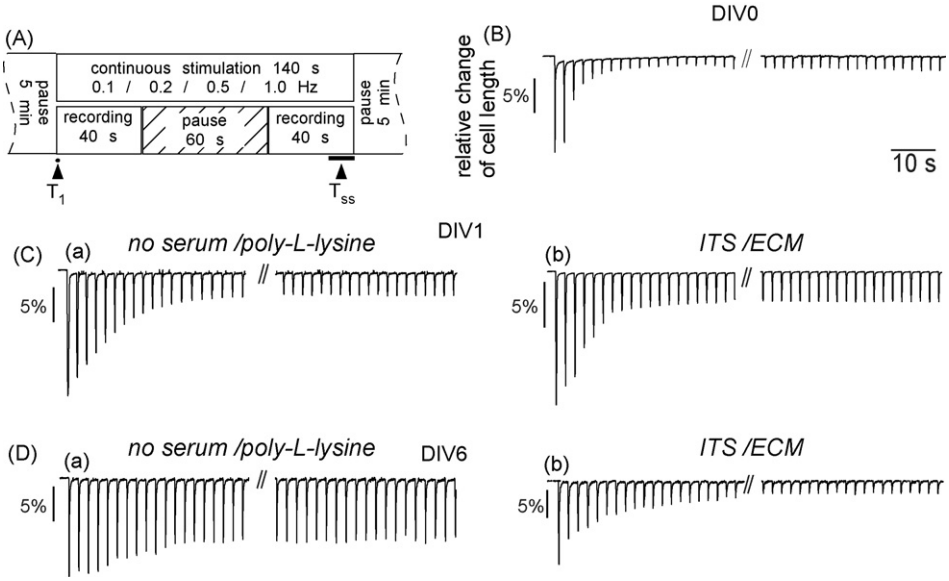

Fig. 4. Post-rest behaviour of contraction in cultured adult rat ventricular myocytes. The basic stimulation protocol for characterising the post-rest behaviour of the cultured myocytes is depicted in panel (A). Panel (B) illustrates the typical time course of cell length changes during such trains of stimulations at DIV0. In panels (C) and (D) cell length changes were plotted for cells at DIV1 (C) and DIV6 (D) that were cultured in serum free/poly-L-lysine (a) or ITS/ECM (b) conditions.

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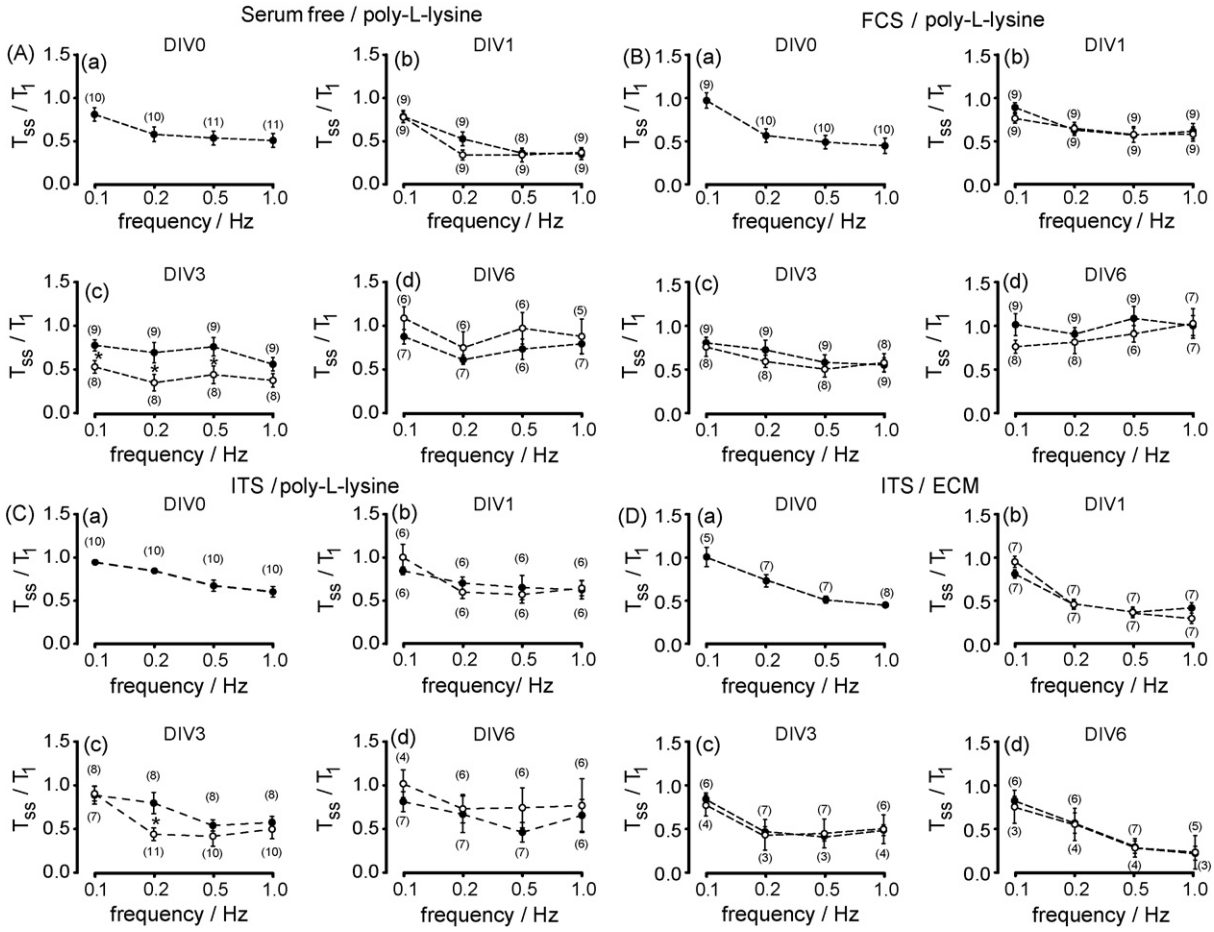

Fig. 5. Frequency dependence of the post-rest behaviour of contraction in cultured adult rat ventricular myocytes. For panels (A–D) populations of cells (taken from between 2 and 6 rat hearts) have been analysed and their post-rest behaviour has been quantified as T_{ss}/T_1 ratios (see Fig. 2A and text for details). This ratio has been plotted against the stimulation frequencies (0.1–1.0 Hz; random order, 5 ms duration). Open and closed symbols represent data from myocytes that had been continuous pulsed (0.2 Hz, 5 ms duration) or not-pulsed, respectively. The numbers adjacent to each data point gives the number of myocytes observed. Pairs of data points with a significant difference have been marked with an asterisk.

ulations (Fig. 5 open symbols) we found no major changes in the contractile behaviour of the myocytes regardless of their other culture conditions. Nevertheless, we observed a significant change at DIV3 with a stimulation frequency of 0.2 Hz for cells cultured in ITS supplemented medium with a poly-L-lysine coating (Fig. 5Cc, marked with an asterisk). The T_{ss}/T_1 ratio displayed a 45% decrease in pulsed myocytes ($n = 11$; non-pulsed cells, $n = 8$). Furthermore, significant differences between paced and non-paced cells were apparent for myocytes cultured under serum free/poly-L-lysine conditions for DIV3 (Fig. 5Ac). Nevertheless, the rather flat frequency dependence was still preserved during pacing.

From these data we concluded that the contractile behaviour at DIV0 was best preserved in a culture medium supplemented with ITS when cells were grown on ECM coated substrates regardless of whether they were continuously paced or not. In the following we conducted a similar series of experiments analysing global Ca^{2+} transients with the Ca^{2+} sensitive fluorescent probe fura-2.

3.5. Calcium–frequency relationship

Figs. 6 and 7 summarise experiments performed under similar experimental conditions as for Figs. 4 and 5 using cells from the same preparations in order to be able to correlate the data with each other. Fig. 6A illustrates the method of fura-2 imaging that we used (for a detailed description see Section 2). Similar to the twitch data presented in Fig. 4A, the global Ca^{2+} transients also displayed post-rest potentiation when measured in freshly isolated rat ventricular myocytes (Fig. 6B for DIV0). We compared time-dependent changes of the Ca^{2+} transient amplitude under conditions of serum free medium and poly-L-lysine coating with the behaviour of cells in ITS supplemented medium and on ECM coating (Fig. 6C and D, a and b, respectively). As a result we also found a loss of post-rest potentiation only in the former condition while under ITS/ECM conditions post-rest potentiation was largely conserved. At DIV6 in the absence of serum the initial post-rest potentiation even turned into a strong post-rest decay (Fig. 6Da).

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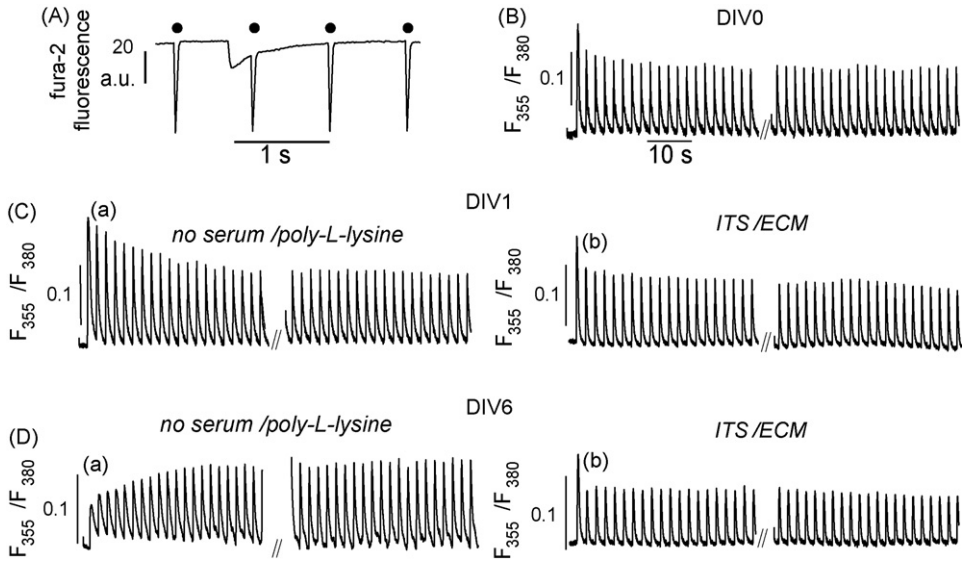

Fig. 6. Post-rest behaviour of Ca^{2+} transients in cultured adult rat ventricular myocytes. Panel (A) illustrates the mode of fura-2 recording (downward deflections marked by a filled circle correspond to the 355 nm excitation images; for further details see text). The typical time course of fura-2 ratio transients during such trains of stimulations at DIV0 is depicted in panel (B). For the stimulation regime see Fig. 2A. In panels (C) and (D) fura-2 ratio transients were plotted for cells at DIV1 (C) and DIV6 (D) that were cultured in serum free/poly-L-lysine (a) or ITS/ECM (b) conditions.

Since in almost 50% of all cells tested at DIV0 the Ca^{2+} transients fused together (relaxation was not complete between the transients leading to a gradual diastolic build-up of the Ca^{2+} concentration) when stimulation frequencies exceeded 0.5–0.7 Hz we omitted the 1 Hz data in the further analysis. All other conditions were similar to those described in Fig. 5. In contrast to the relationships of the twitch amplitude the height of the Ca^{2+} transients only displayed a modest post-rest potentiation with a basically flat frequency dependence at DIV0 (Fig. 7Aa). This flat amplitude–frequency relationship was basically preserved for all DIVs and for all culture conditions with the exception of a sole set of conditions. Here, the myocytes at DIV6, that were paced continuously in the absence of any medium supplement on poly-L-lysine coating, displayed a dramatic shift from modest post-rest potentiation at 0.1 Hz to a significant post-rest decay at 0.5 Hz (open symbols in Fig. 7Ad). Similarly to the results we obtained for the twitch measurements (Fig. 5) continuous pacing did not make any difference to the frequency relationships at any DIV nor under any culture condition apart from the ITS/poly-L-lysine combination at DIV3 (Fig. 7Cc).

Thus, myocytes cultured in ITS-supplemented medium and growing on ECM coated substrates most closely retained their morphology, contractility and Ca^{2+} handling when compared to their properties at DIV0.

We thus conducted the final series of experiments to investigate whether cardiomyocytes cultured under ITS/ECM conditions were a good system to perform long-term expression of exogenous proteins. From our fura-2 data we knew that under our culture conditions, Ca^{2+} handling was largely conserved through the 1-week period of culturing. We thus tested expression of a genetically coded Ca^{2+} indicator by adenoviral gene transfer (inverse pericam as originally described by Nagai et al. [10]).

3.6. Inverse pericam expression and Ca^{2+} measurements

The freshly isolated ventricular myocytes were infected with the virus 1 h after plating and first fluorescence could already be recorded within 24 h. Typically we started experiments 18 h after transfection. Already at that early stage, more than 95% of all viable myocytes displayed sufficient levels of fluorescence for recording Ca^{2+}-dependent fluorescence changes (data not shown). Fig. 8A exemplifies such changes as recorded in response to electrical stimulations. In all cells analysed at DIV1 we could record a maximal relative fluorescence change of $25 \pm 3.1\%$ ($n = 65$). Since excitation of inverse pericams does not require the application of UV light we tested the possibility to perform long-term recordings as depicted in Fig. 8B. We designed an electrical stimulation regime employing constant pulsing at 0.1 Hz and optical recording for 40 s periods separated by 6 min without light excitation. For the recordings in Fig. 8B we continued this stimulation regime for a total of 90 min without a detectable loss in pericam self-ratio amplitude or signal quality. During the same time period the absolute inverse pericam fluorescence was decreased by less then 15%, most probably due to bleaching. In some experiments we recorded for more than 2 h with a similar experimental regime without a noticeable decrease in signal quality (data not shown).

4. Discussion

The aim of this study was to develop and explore a culture system for adult rat cardiac myocytes and procedures

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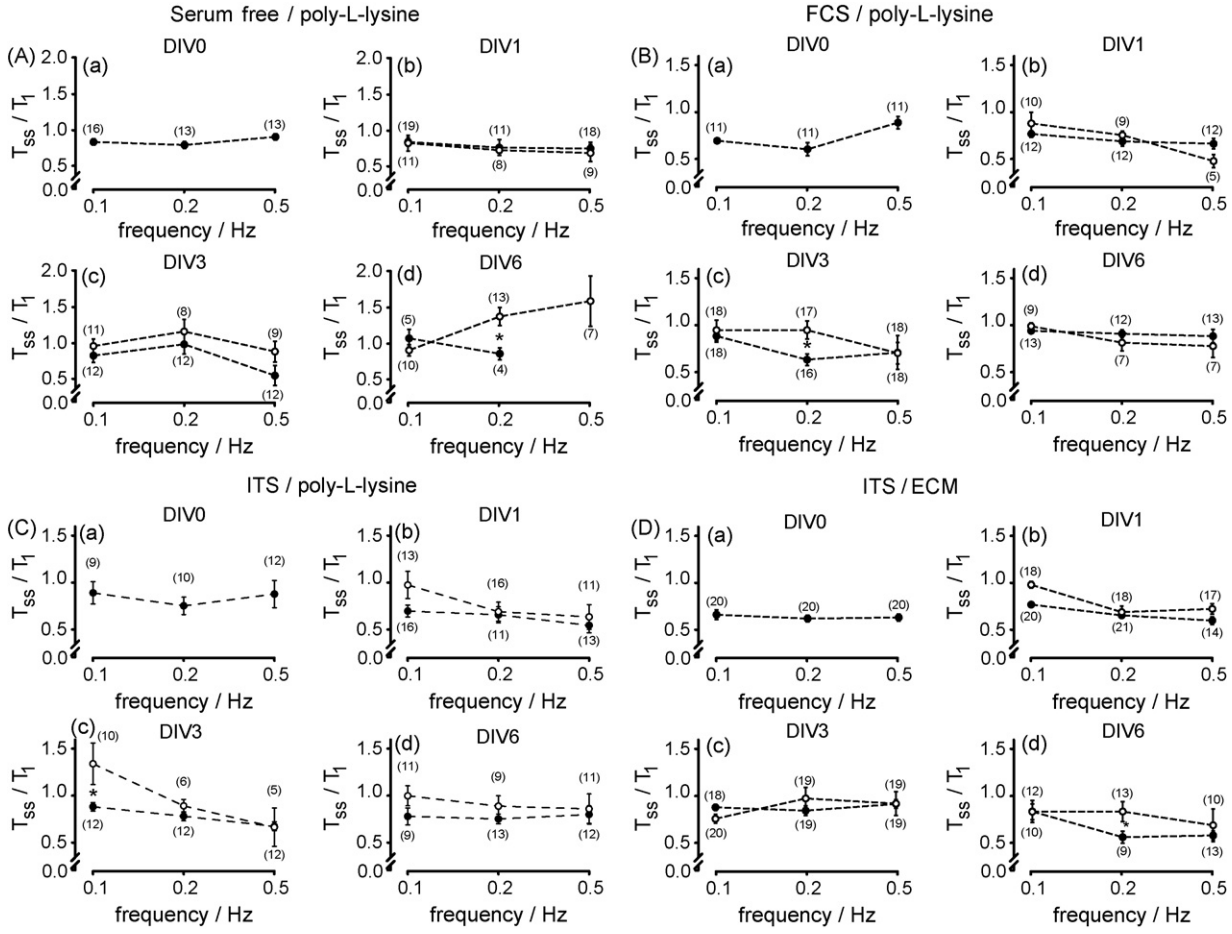

Fig. 7. Frequency dependence of the post-rest behaviour of Ca^{2+} transients in cultured adult rat ventricular myocytes. For panels (A–D) a population of cells (taken from 2 to 6 rat hearts) have been analysed and the amplitude ratios T_{ss}/T_1 have been plotted against the stimulation frequencies (0.1–0.5 Hz, random order, 5 ms duration) for various culture conditions as depicted in each panel. Open and closed symbols display mean values for the continuously pulsed and non-pulsed myocytes respectively (0.2 Hz, 5 ms pulse length). The numbers adjacent to each value give the number of myocytes analysed. Pairs of data points with a significant difference have been marked with an asterisk.

that allow extended experimental manipulation of these cells under conditions of diminished dedifferentiation. For that we setup an experimental system for a long-term culture of adult cardiac myocytes that largely suppressed de-differentiation, best maintained the morphological and physiological properties of freshly isolated cells and allowed (to our knowledge) for the first time long-term expression of a genetically encoded Ca^{2+} indicator.

One of the biggest problems with long-term cultures of adult cardiac myocytes is the rapid development of morphological and physiological dedifferentiation. The structural changes occurring during extended culturing times of rat ventricular myocytes were studied extensively (e.g. [14,15]). In parallel there were numerous attempts to modulate culture conditions towards minimising dedifferentiation. Such approaches included omitting or substituting the medium supplement FCS (e.g. [11]) and electrical pacing of the adult cells (e.g. [9]). All of those approaches have provided progress towards conditions allowing extended culture periods and reduced dedifferentiation of the adult cells.

We omitted serum from our medium and substituted that by an ITS mixture. In addition, we coated the substrates (plastic and glass) with ECM. Both steps revealed major improvements in both, the number of viable cells (i.e. the loss of viable cells was greatly reduced between DIV0 and DIV6) and the preservation of cell morphology, subcellular microarchitecture (contractile filaments) and physiology (contractility and Ca^{2+} handling) of the cells over the time course of culturing.

As described before, also in our hands the presence of serum in the culture medium appeared to promote dedifferentiation resulting in a flattened morphology. Interestingly, similar shapes are “normal” for cardiac cell lines such as the H9C2 cell line investigated earlier (e.g. [16]). We found that simply omitting the serum supplement is slowing down the dedifferentiation process (see also [11] for rabbit cardiomyocytes) and more cells survive the culture period with an elongated cell body. Unfortunately, adult rat cells cultured under these conditions displayed a progressively increasing number of subcellular vacuoles and/or vesicles, a gradual loss

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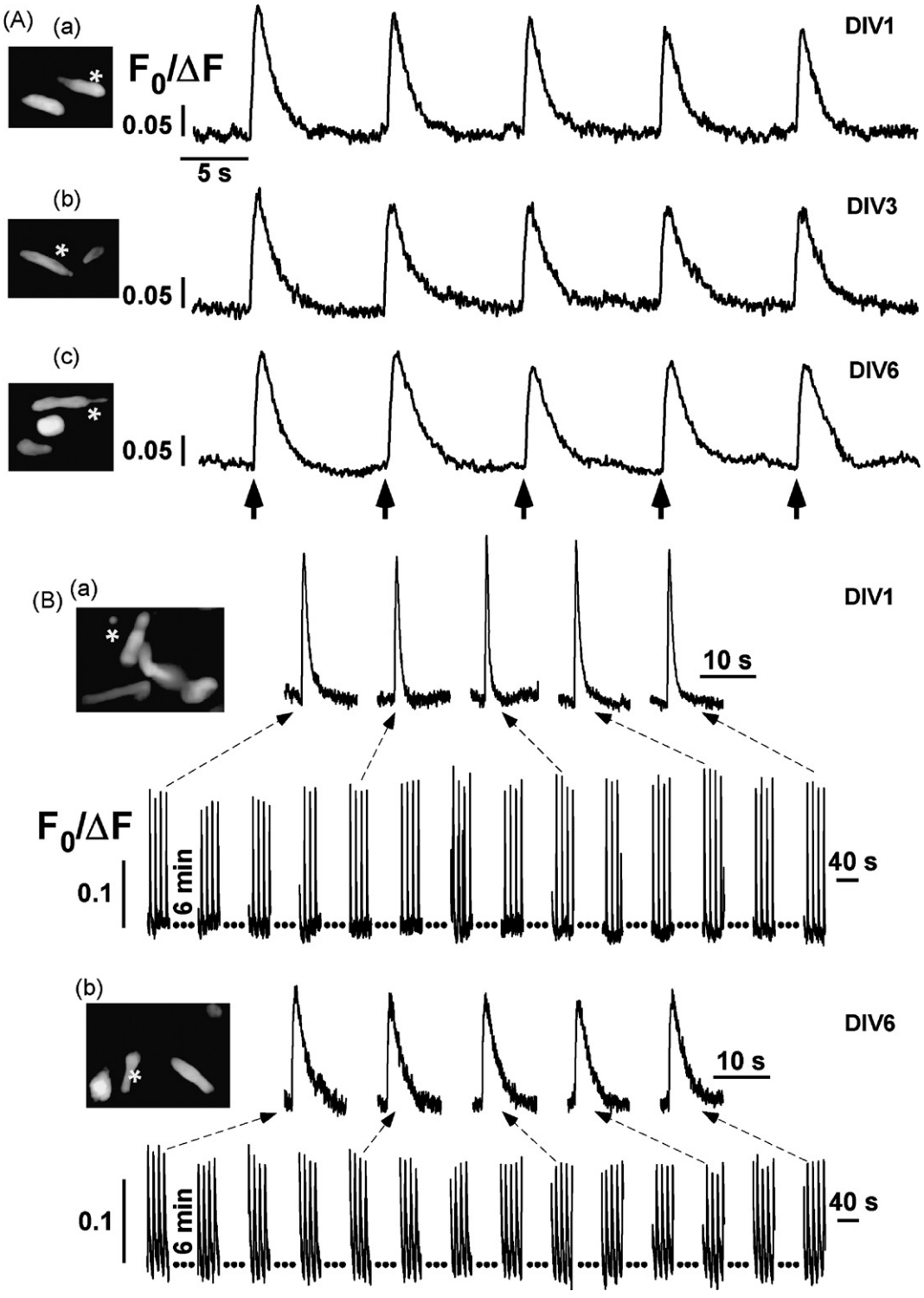

Fig. 8. Fluorescence transients of inverse pericam in cultured rat ventricular myocytes after adenoviral gene transfer. Panel (A) depicts typical self-ratio traces of the inverse pericam fluorescence recorded from cultured myocytes at the time points given. Panel (Ac) illustrates the stimulation regime used (black arrows indicate electrical impulses, 5 ms duration). Cell images are added for each example and the cell used for calculation of the ratio traces has been marked with a white asterisk. Panel (B) shows ratio traces of adult ventricular myocytes 1 day (Ba) and 6 days (Bb) after virus infection. Dashed arrows depict recording periods for which exemplified individual ratio transients have been replotted. In the fluorescence images an asterisk marks the myocyte from which the ratio was calculated. Here, we calculated $F_0/\Delta F$ (F_0—fluorescence at the beginning of the recording at rest) to obtain positive changes of the ratio, since the inverse pericam displays a decrease of the fluorescence with increasing Ca^{2+} concentrations. Traces shown here were typical for all cells analysed under these experimental conditions ($n > 60$ from three rat hearts).

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of cross-striation and alterations of their post-rest behaviour. From such findings we concluded that this culture condition was in fact not optimal. In contrast ITS-supplemented medium greatly improved the conservation of the cellular properties found at DIV0 whether the cells were seeded on poly-L-lysine or ECM coated substrates, whereby the latter condition gave even better results.

In the vast majority of conditions, there was neither a beneficial nor a detrimental effect of electrical pacing unlike the report by Berger et al. [9]. This held true for both physiological parameters that we analysed, the contractile performance and Ca^{2+} transients in response to electrical stimulation as well as the stimulation frequency dependence of both parameters. Nevertheless, this does not exclude the possibility that certain culture conditions combined with particular stimulation protocols might increase or decrease survival of the cells or their morphological/physiological state. Interestingly other studies described hypertrophic responses of myocytes when pacing frequencies were raised to higher stimulation frequencies in adult myocyte cultures (3 Hz; [17]). This is puzzling since these stimulation rates are rather physiological for rodent hearts (3–10 Hz) at 37 °C.

We made the observation that the absolute maximal cell length change was progressively decreasing over the culturing time. This reduced twitch amplitude can most likely be attributed to a progressively increasing mechanical interaction between the cells and the substrate. Indeed, during regular washing of our culture flasks relative loss of cells was reduced at later DIVs, a finding that might hint to a tighter substrate interaction of the myocytes (data not shown). Such an increased mechanical coupling will simply decrease absolute twitch amplitudes despite a constant contraction force. A mechanism that might be responsible for this was recently found and described the production of extracellular matrix proteins and modulation of the matrix by myocytes themselves [18]. Therefore, we investigated our cells using their post-rest behaviour at various stimulation frequencies: (i) post-rest contractile behaviour of isolated myocytes is attributed to their ability to adjust Ca^{2+} handling to changes (frequency, rest) of the excitation–contraction coupling [13], (ii) the rational behind this protocol was to work with relative shortening changes and to avoid individual differences and changes of cell-substrate interactions as described above, and (iii) to compare an entire range of frequency dependences rather than selected electrical stimulation regimes.

Genetic manipulation of adult cardiac myocytes is often limited to short term procedures and to generation of genetically modified donor animals. While the latter is a very elegant way of introducing proteins to or knocking proteins out from cardiac myocytes, the generation of genetically modified animals is expensive and tissue specific, inducible genetic manipulation is not an easy routine work. Thus, genetic manipulation of isolated cardiac myocytes might be a feasible intermediate step towards genetically modified animals. Traditionally, genetic manipulation of cardiac myocytes has been performed on neonatal ventricular cells from the rat (e.g. [19]). Neonatal cells can be transfected with traditional means (i.e. commercially available transfectants [20]) but in adult cells the yield of such an approach is usually <10%. Better expression or knock-out rates can be achieved with viral gene transfer systems, e.g. adenovirus. Unfortunately, results obtained from neonatal systems are often difficult to transfer to the adult situation, due to significant developmental differences in the expression pattern and signalling. Therefore, it is desirable to perform such experiments on adult cardiac myocytes. Indeed, there are some examples where genetic manipulation, e.g. with an adenoviral system has been applied successfully (e.g. [21]). Mostly, such approaches have been limited to short-term expression (1–2 DIV) and have thus avoided unwanted dedifferentiation of the cells. Nevertheless, longer expression would be highly desirable since many effects of genetic manipulations and/or stimulation regimes will require longer culture periods.

To our knowledge we described for the first time the expression of a genetically encoded Ca^{2+} sensor (inverse pericam) in adult rat ventricular myocytes for extended periods of time. In our hands, the myocytes cultured under ITS/ECM conditions were not negatively affected by the gene transfer through an adenovirus. Expression levels were already high enough for fluorescence recording less than 24 h after infection and throughout our culture period of 7 days expression levels were steadily increasing. This will certainly require additional investigations and titration of the best MOI for rapid onset of fluorescence and stable expression levels. In less than 24 h expression levels allowed extended recording regimes of Ca^{2+} transients without detrimental effects in the signal to noise ratio. In some experiments we were able to record for more than 2 h and the recording was not limited by bleaching or a decrease in the cell quality. Moreover, the frequency of data acquisition was not reduced in favour of a prolonged recording time; we collected images at a rate of 50–60 frames/s that allowed, e.g. the recoding of Ca^{2+} waves (data not shown). Thus, extended expression of genetically encoded Ca^{2+} sensors is a promising approach for long-term, continuous observation of Ca^{2+} handling in isolated cardiac myocytes, especially when working on species for which an appropriate transgene does not exist. A potentially interesting transgenic mouse expressing a Ca^{2+} indicator was introduced recently [22].

In the present report we have introduced a potentially important approach for culturing rat ventricular myocytes combined with expression of exogenous proteins for extended periods of time. Such an approach will allow further explorations of *in vitro* models for studying long-term signal transduction in cardiac myocytes enabling quasi continuous supervision of physiological and morphological parameters such as contractility and Ca^{2+} handling. We envisage our report to be a foundation for the further development of high-content screening systems [23] employing adult cardiac myocytes because the application of adenoviral gene transfer allows the application of genetically encoded reporters such as Ca^{2+} sensors but also sensors of other sig-

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nal transduction processes (e.g. phosphorylation) in cardiac myocytes.

Acknowledgements

This work was supported by Deutsche Forschungs Gemeinschaft (SFB 530 TP B6), Graduate Research School "Cellular Regulation and Growth" (GRK 377/3) and Saarland University (ZFK and HOMFOR). We are grateful to Anne Vecerdea, Tanja Buhles, Dr. Klaus Neumann and Jörg Sauerbaum for their excellent technical support. We also would like to thank Rod O'Connor (The Babraham Institute, UK) for the initial version of the software "Cardiac Stimulator".

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