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Review

Cultured adult cardiac myocytes: Future applications, culture methods, morphological and electrophysiological properties

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Abstract

Isolated adult cardiac myocytes maintained in primary culture have been used as a model of the adult myocardium for 20 years. With the recent advances and current interest in using molecular biological techniques to investigate cardiac physiology, culturing myocytes is becoming an increasingly important technique. Acutely isolated myocytes do not remain viable for the time needed for the changes in gene expression to occur, and therefore it is necessary to maintain myocytes in culture. The aims of this review are: (1) To describe a method for isolating and culturing myocytes in serum-free medium. This section is targeted at new researchers in the field, with particular emphasis on aspects of the isolation procedure which are important for optimising myocyte culture. (2) To review current knowledge of how contractile, electrophysiological and morphological properties of adult myocytes are preserved in culture. Over the past 5 to 10 years significant advances have been made in developing novel techniques which help maintain the in-vivo properties of myocytes in culture. Efficient methods for transporting exogenous genes and anti-sense oligonucleotides into adult myocytes are now available. We anticipate that in future these advances will make cultured myocytes more attractive for use in biophysical and molecular investigations of cardiac physiology. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cardiac myocytes; Culture; Morphology; Electrophysiology; Contraction; Molecular biology

1. Introduction

1.1. Developments in heart cell culture

Heart tissue was first put in culture over 80 years ago [1]. Burrows (1912) placed pieces of explanted embryonic chick hearts in culture and observed that single individual cells migrated away from the explants. It was another 43 years before heart tissue was dissociated and maintained in culture as *single cells*, first by Cavanough (1955), who isolated and maintained chick embryo myocytes [2] and then by Harary and Farley (1960) who were the first to culture mammalian myocytes [3]. Adult cells are strongly physically connected by intercalated discs and extracellular matrix, and therefore are more difficult to isolate. It was not until 1976 that a technique for isolating viable adult ventricular myocytes was first described [4], and adult cells were first cultured in 1977 [5]. The majority of long-term studies using cultured myocytes have been performed on embryonic and neonatal myocytes (e.g. Refs. [3,6–10]). However, with respect to the in-vivo myocardium, it may be preferable to study *adult* myocytes. Changes in expression of ion channels and contractile protein isoforms during development may make it problematic to extrapolate results from embryonic and neonatal cultured myocytes to the fully differentiated adult myocardium. Early developments in the culture of adult myocytes have been reviewed previously [11,12]. However, in recent years contractile and electrophysiological properties have been investigated, novel techniques for improving preservation of myocyte properties in culture have been developed (e.g. Refs. [13,14]) and cultured adult myocytes have been used for molecular biological experiments (e.g. Refs. [15,16]). The aims of the present article are: (1) To describe a method developed in our laboratory for culturing adult *rabbit* myocytes (we work primarily with rabbit myocytes, but the techniques are also applicable to rat,

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guinea-pig and cat). (2) To review current knowledge of adult myocyte properties in culture. Since electrophysiological and contractile properties are central to the function of myocytes in-vivo and are useful for assessment of cell viability [11], we devote a section to describing these properties in culture. Discussion of these facets of myocyte culture will be placed in the context of the potential of cell culture for several applications, and in particular molecular biological investigations.

1.2. Why use cell culture?

For electrophysiological experiments, myocyte culture has many of the advantages of acutely isolated myocyte preparations. It offers a homogenous population of single cells, which are easy to visualise and manipulate in the recording chamber. The properties of myocytes can be examined in a controlled environment, without interference from non-myocytes (such as fibroblasts and endothelial cells). Myocyte culture has other potential advantages. (1) It provides myocytes with time for damage sustained during the enzymatic isolation procedure to be repaired, including re-expression of damaged membrane proteins [17], such as receptors and ion channels. (2) Cultured myocytes can be used for longer term studies which are not possible using acutely isolated myocytes. In our experience, acutely isolated adult myocytes typically remain in good condition for only 8 to 12 h. In contrast, cultured adult myocytes can remain viable for days to weeks, depending on the culture conditions used (e.g. Ref. [18]). Longer term studies may involve manipulating the environment in which cells are maintained, allowing separation of humoral influences on cardiac myocytes from the large number of other stimuli that exist in-vivo. (3) It allows manipulation of protein expression in single myocytes using molecular biological techniques. These techniques are critically dependent on maintaining myocytes in culture to preserve the viability of the cells while the changes to protein expression occur [19,20]. (4) In future, the ability to maintain cardiac myocytes in culture may help resolve difficulties in obtaining human or primate heart tissue. In addition to experimental advantages, if myocytes can be preserved in culture there are also ethical and practical reasons for culturing heart cells, since it may lead to sacrifice of fewer animals, and simultaneously reduce the time and money spent isolating cells.

Clearly, despite potential advantages, cultured myocytes are not a substitute for whole animal or intact heart preparations. Myocytes adapt to the culture environment and therefore cannot be considered to be in a stable steady-state. Reorganisation of cytoskeletal components and contractile proteins occurs as cells adapt from a three dimensional (3D), in-vivo environment to the two dimensional (2D) ''culture dish'' environment [21–23]. It is not clear to what extent ''de-differentiation'' (i.e. reversion to a foetal phenotype) occurs. The expression of β-myosin heavy chains (βMHCs), which have been used as markers of de-differentiation (since βMHCs are absent in adult, but present in immature myocardium), remains stable in some cultures [24] but increases in others [25,26]. Many in-vivo properties of adult heart muscle appear to be observed, even in the most morphologically altered states [27]. In considering the merits of cultured myocyte preparations, therefore, cultured myocytes may *complement* (rather than replace) acutely isolated myocytes, and may represent a useful model of adult in-vivo cardiac tissue for different studies, especially those of extended duration.

1.3. Adult ventricular myocytes in culture

There are two basic methods for culturing adult ventricular myocytes as defined originally by Jacobson and Piper (1986) [11]. The first maintains myocytes in a serum-supplemented medium, usually without cell attachment to the culture surface (e.g. Refs. [5,28,29]). Under these conditions myocytes remain in suspension and become rounded, losing their in-vivo rod shaped morphology [28]. After 2 to 4 days in suspension the cells attach to the culture surface and start to ''spread'', sending out pseudopodia-like projections in different directions [28]. During the spreading process, changes in ultrastructure indicative of differentiation occur, and so for this reason the culture technique is known as the ''redifferentiation'' method [11]. The myocytes re-develop an extensive transverse (T) tubule system, sarcoplasmic reticulum (SR) and mitochondria [11,30]. Myocyte cultures tend to become spontaneously contractile over time [5,11,27,30,31]. If contacts form with neighbouring cells, synchronous contraction of all cells occurs, indicating that functional gap junctions have been re-expressed [11].

The second culture technique has been termed the ''rapid attachment'' method [11]. The culture surface is pre-treated with attachment factors and the myocytes are plated out in a serum-free medium. Cells adhere to the culture surface within 3 h of being plated out [32]. Using this technique, the cells retain the rod-shaped and striated appearance of acutely isolated myocytes for 1 to 2 weeks (e.g. Ref. [33]), in contrast to the redifferentiation method, where myocytes lose their rod-shape morphology.

One advantage of the ''redifferentiated'' culture model is that myocytes can be maintained for weeks to months (e.g. Refs. [30,34]). However, the myocytes are structurally different from in-vivo myocardium, and often exhibit spontaneous contractions. These are characteristically different to contractions often associated with a high SR Ca content [35,36] or loss of Ca tolerance after the isolation procedure. The mechanisms of spontaneous contractions observed in redifferentiated cultures may be comparable to the contractile activity of intact foetal tissue, or may result from catecholamines and other positively inotropic agents contained in serum. Either way, spontaneously active

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cultures may be functionally different from normal in-vivo adult myocytes. Furthermore, myocytes cultured using the redifferentiation method usually require serum in the external medium. Serum contains unknown concentrations of many growth factors and hormones, each of which may have an unquantifiable effect on myocytes. For these reasons, it may be preferable to culture cells in a defined medium [12].

Myocytes cultured using the ''rapid attachment'' method do not contract spontaneously, or require serum, and remain viable for up to 14 days [37,38]. Homogeneous populations of >95% rod-shaped cells can be obtained routinely. The serum-free conditions do not support non-myocyte proliferation and so anti-mitotic agents are not needed. As techniques improve, it may be possible to increase the length of time that myocytes remain viable using the rapid attachment method, leading to better long term in-vitro models of the adult myocardium. This article will concentrate primarily on myocytes cultured using the rapid attachment method, although studies from myocytes maintained in serum-supplemented medium (still used routinely by some groups) have been included for comparison.

2. Methods

2.1. Myocyte isolation techniques

The nature and quality of the myocyte isolation procedure may be the single most important and influential factor for successful cardiac myocyte culture. Although culture conditions may provide myocytes with a favourable environment to recover from trauma sustained during isolation, in our experience the survival of myocytes in culture often seems correlated with the quality of the isolation. The aim of this section is to highlight aspects of myocyte isolation which appear important for successful myocyte culture. It is not intended to be a comprehensive review of literature on cell isolation, which is covered elsewhere [12,39–41] (and which anyway varies considerably between laboratories).

2.2. Preliminary considerations

The animal species from which myocytes are isolated is likely to depend primarily on the experimental parameter of interest. However, there is evidence that myocytes of different species may adapt to culture conditions in different ways. Some change morphology in culture more rapidly (e.g. rabbit [22] compared to feline myocytes [42]). The number of myocytes isolated in a procedure may also be an important consideration. Rat, cat and rabbit are the most common species used for heart cell culture. We have used the method described below for isolation of adult rabbit myocytes. However, our laboratory also uses a similar method to isolate rat and guinea-pig myocytes, adjusting solution volumes with respect to coronary perfusion rate, and finding optimal enzyme perfusion times for each species.

Attachment of myocytes to the culture surface influences cell morphology [38,43,44]. It is possible that interactions with the culture surface may modulate other properties, including membrane currents and contraction. The most widely used attachment factor is laminin (e.g. Refs. [24,42,45]; Sigma or Collaborative Research). Laminin is reliable, easily applied to plates, and myocyte attachment is rapid. Techniques for using laminin will be described in more detail below. However, if needed in large quantities, laminin may be prohibitively expensive. Type IV collagen (Gibco, Collaborative Research) is also effective for adult myocyte attachment, but collagen type I and III give poor results [46]. Diluted foetal calf serum (FCS; Gibco) was the first attachment factor to be used for adult heart cells [32]. It is inexpensive and can be effective [38]. However, it requires time consuming testing to find an effective batch, takes 5–15 h to apply and may give variable results from one isolation to another (Mitcheson, personal observations).

All myocyte isolation techniques for cell culture use a low Ca solution to disrupt intercellular connections, followed by enzyme digestion to break down the extracellular matrix, and finally mechanical agitation to separate the single cells. Finding particular enzyme batches which dissociate myocytes without causing permanent damage, seems critical. Crude collagenase alone, or in combination with protease or hyaluronidase (or both), are the most commonly used enzyme combinations. Pure collagenase and protease do not produce good results, and therefore different batches of crude forms of these enzymes need to be tested. There appears to be no ideal technique for evaluating the performance of enzyme batches. One quick and convenient method is to count the total number of rod-shaped myocytes and calculate the percentage of rod-shaped to rounded myocytes, both immediately after isolation and at intervals during cell culture.

Microbial infection of cultures rapidly removes essential constituents from culture media, alters the pH balance of the media, and may introduce active substances that are harmful to myocytes. Bacterial infections can be eradicated by adding antibiotics to the culture medium (e.g. streptomycin and penicillin are routinely used). However, prevention is the best procedure for dealing with fungal or yeast contamination, since fungicides are highly toxic to myocytes. The isolation apparatus can be kept in a class II flowhood or laminar flow cabinet to prevent airborne contaminants from infecting solutions during the isolation procedure. Fig. 1a shows isolation apparatus that can be arranged to take up minimum space in a flowhood. It is important to use the smallest components possible and to check that the flowhood performance is not compromised by excessive air turbulence. Other possible procedures

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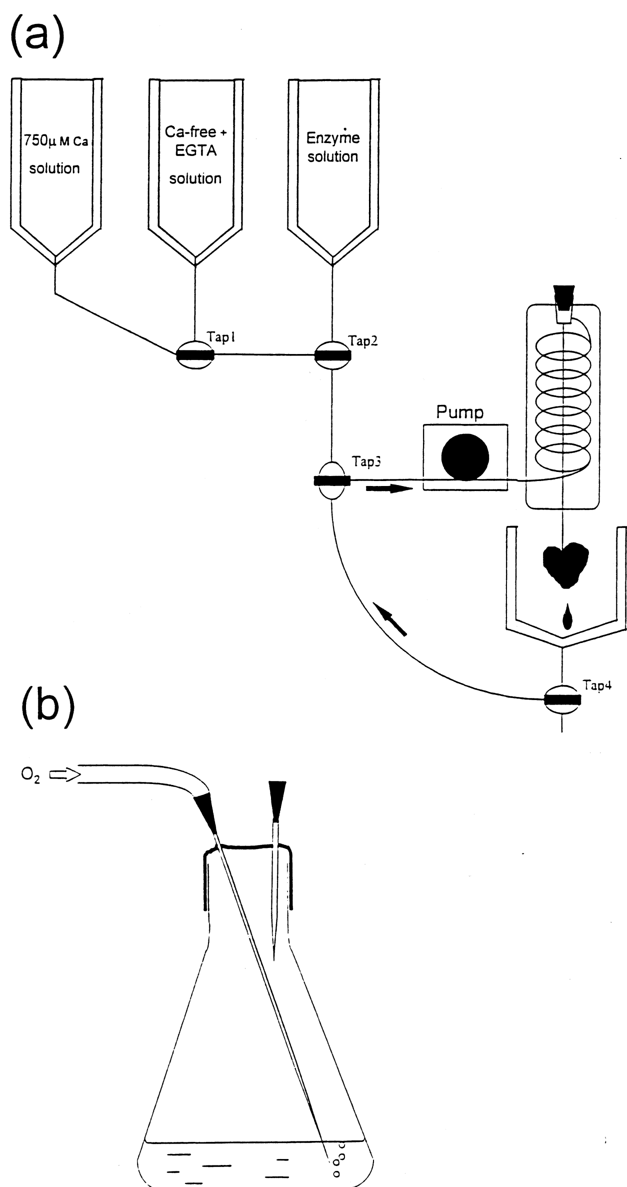

Fig. 1. (a) Apparatus used to isolate single cardiac myocytes at 37°C. (b) Shaker apparatus, designed to oxygenate cells and prevent microbial infections in a non-sterile environment. Taken from Ref. [47] with permission.

which may help prevent infections are: (1) A thorough rinse of the isolation apparatus with 70% alcohol, followed by at least two rinses with sterilised de-ionised water immediately before heart perfusion. (2) Apparatus that can be easily taken apart, sterilised, and reassembled just before starting the isolation. All precautions mentioned above are best used in conjunction with filtering of solutions and other routine sterile techniques.

2.2.1. Isolation apparatus

Using the apparatus shown in Fig. 1a, hearts may be perfused retrogradely through the aorta, and thus through the coronary arteries, which supply the muscle of the ventricles [47]. The same apparatus, with modified solution flow-rates and cannulae, can also be used for rat and guinea-pig isolations. The apparatus consists of:

1. Three top solution reservoirs which hold a minimum of 300 ml and are temperature controlled by passing warmed water from a water bath through the glass jackets (''water-jacketed'').
2. A glass-coil heat exchanger fitted with cannula and bubble trap to prevent gas bubbles from entering the heart and blocking coronary circulation.
3. A water-jacketed reservoir which surrounds and warms the heart and collects solutions for recirculating back into the heart (indicated by arrows, Fig. 1a).
4. A variable speed peristaltic pump which can operate at solution flow-rates of up to 100 ml min^{-1}.
5. Bubblers for oxygenating solutions in reservoirs.
6. Three-way taps for rapid switching between solutions.

2.2.2. Isolation solutions

The basic isolation perfusion solution (Solution A) made with Milli-Q grade water and high purity reagents (e.g. ''Aristar'' grade from British Drugs House) contains (in mmol l^{-1}): NaCl, 130; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 23; glucose, 21; taurine, 20; creatine, 5; $MgCl_2$, 5; Na pyruvate, 5; KCl, 4.5; NaH_2PO_4, 1; pH 7.3. The solution is filtered (0.2 μm filters) to remove microbes and small particles. Solution A is the stock for the four other solutions used during the isolation procedure: (1) 750 μM Ca solution (300 ml Solution A+750 μM Ca). (2) Ca free+EGTA solution (300 ml Solution A+3.3 μM EGTA). (3) Enzyme solution (80 ml Solution A+100 μM Ca, 1 mg ml^{-1} crude collagenase (Worthington Type 1A), and 0.1 mg ml^{-1} protease; Sigma Type XIV). (4) ''Enzyme washout'' solution (220 ml Solution A+100 μM Ca).

2.2.3. Culture medium

The basic, ''non-supplemented'' medium routinely used to culture adult heart cells is bicarbonate buffered Medium 199 (e.g. Refs. [24,37,48]). Medium 199 contains all amino acids (except glutamine), vitamins and the following ionic constituents (in mmol l^{-1}): $CaCl_2$, 1.8; NaCl, 116; Na acetate, 0.6; $NaHPO_4$, 1; KCl, 5.3; $MgSO_4$, 0.8. Common additional supplements are (in mmol l^{-1}); creatine, 5; taurine, 5; L-carnitine, 2; pyruvate, 2.5; and insulin 10^{-7} M (e.g. Refs. [13,24]). To prevent bacterial infection all media contain 50 I.U. penicillin and 50 μg ml^{-1} streptomycin. The cells are maintained under sterile conditions in an incubator in a 5% CO_2–95% air atmosphere at 37°C.

2.2.4. Pre-treating culture surface with attachment substrate

Tissue grade plastic (e.g. Falcon or NUNC) or glass can

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be used. Glass coverslips are washed in 70% ethanol, rinsed in water and autoclaved before use. Etching the glass with nitric acid has been proposed to aid optimal attachment [12], but may not be strictly necessary. Laminin is diluted to a final concentration of between 1–5 μg ml^{-1} in a volume of phosphate buffered saline or culture medium which adequately covers the culture surface. The laminin is applied to the culture plates at least 30 min before plating out cells (or longer if working at less than 37°C).

2.3. Isolation procedure

The animal is killed humanely by cervical dislocation and the heart excised, taking care to remove the pericardium. Blood is removed from the coronary vessels by gently squeezing the heart a few times in cold (4°C) 750 μM Ca solution. The aorta is tied onto the cannula and the heart perfused with 750 μM Ca solution. After 2 min the solution is switched to Ca free+EGTA solution for 5 min. After this the heart is perfused with enzyme solution for between 8 and 14 min. When the enzyme starts to digest the heart, fluid leaving the heart becomes more viscous and is collected in the chamber surrounding the heart and recirculated. At the end of the enzyme digestion, the enzyme solution is washed out of the heart with 100 μM Ca solution for 5 min, after which the heart is cut off the cannula and the atria and aorta dissected away. On a sterile petri dish, the ventricular tissue is chopped with small scissors. The tissue is placed in 50 ml of 100 μM Ca solution in a shaker flask and agitated gently at 37°C to mechanically dissociate the myocytes. Fig. 1b shows shaker apparatus which allows digested tissue to be oxygenated in a waterbath outside the flowhood, without risk of fungal infection. Myocyte suspensions may be damaged by excessive bubbling and therefore oxygenation should be gentle. After a 5 to 10 min period of shaking, the myocytes are filtered through nylon gauze (200 μm mesh). The myocytes are centrifuged at 50 *g* for 1 min and the supernatant replaced first by 500 μM Ca solution, and then by 1 mM Ca solution. The mild centrifugation step discards the majority of non-myocytes and dead or hypercontracted myocytes. Some laboratories find it useful to further enrich the fraction of rod-shaped myocytes by centrifugation through 4% bovine serum albumin or a density-gradient formed with Percoll (Pharmacia). Finally the cells are resuspended in culture medium and plated.

2.4. Myocyte culture

2.4.1. Plating of myocytes

To plate the myocytes at an appropriate density, a small sample are counted using a haemocytometer while in 1 mM Ca solution. The appropriate number of cells are centrifuged and then resuspended in the volume of culture medium needed to give adequate coverage of the culture surface. Myocytes can be plated at densities of up to 10^4 *rod-shaped* cells cm^{-2}. To get a high percentage of myocytes to attach to the plates it may help to follow these precautions. (1) Plate at the correct cell density. If the density of cells is too high, cells clump together, do not attach firmly to the plate and large numbers are lost during the first medium change (personal observation). (2) Plate cells quickly after isolation as extracellular Ca promotes clumping of the myocytes, and thus loss of cells during medium changes [12]. (3) Alter isolation conditions, or use a centrifugation enrichment technique, to get a high percentage of rod-shaped to rounded myocytes. A low percentage of rod-shaped cells prevents effective cell attachment, presumably because the rounded up cells prevent the rod-shaped myocytes obtaining access to the culture surface.

2.4.2. Medium changes

Once myocytes have been plated out they are left to attach for 4 h. After this time any cells which have not attached can be removed by gently changing the medium. Cultured myocytes are sensitive to shear forces and medium turbulence, and therefore medium changes must be performed slowly and carefully. After the first medium change, subsequent medium changes are carried out every 3 days. Medium is warmed and equilibrated with 5% CO_2 beforehand.

One advantage of culturing myocytes on attachment factors is that rounded and hypercontracted myocytes do not attach to the culture surface. Therefore after careful medium changes, >95% of myocytes remaining are rod-shaped and viable. Few non-myocytes are plated out and, in addition, the serum and glutamine free conditions do not support cell division, avoiding the problems of contamination of myocyte cultures by rapidly dividing non-myocytes. If the medium is supplemented with serum, then non-myocytes (in particular fibroblasts) may overgrow the cultures in 2 to 3 weeks. This can be prevented by using anti-mitotic agents such as cytosine-β-D-arabinofuranoside (5 μM).

3. Characteristics of cultured myocytes

3.1. Cell shape and ultrastructural morphology

Cell shape and morphology are intimately linked with some aspects of cell function (such as excitation–contraction coupling [49]). Therefore monitoring these properties may give indications of physiological changes that are occurring. Fig. 2a shows representative photographs of rabbit myocytes over time in culture. Features typical of acutely isolated (Day 0) cells were the "brick" or "rod" shape with rectangular "stepped" ends and clear cross-striations. After 1 day (Day 1) in culture the cells were still rod-shaped with clear cross-striations; however, the ends

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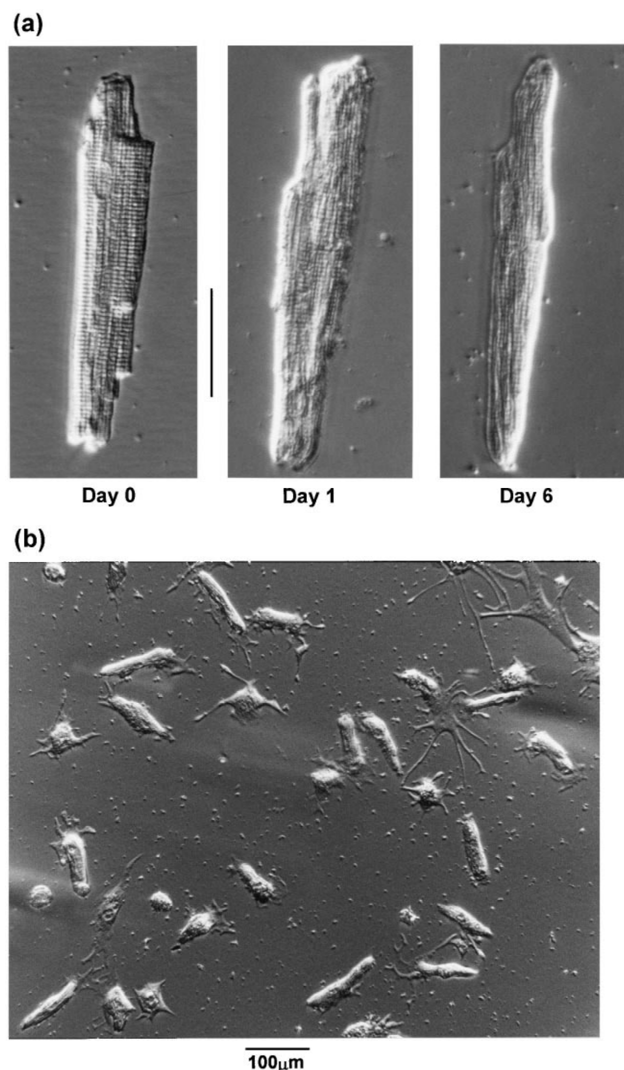

Fig. 2. (a) Typical phase contrast micrographs of rabbit ventricular myocytes immediately after isolation (Day 0), after 24 h in culture (Day 1) and after 6 days in culture (Day 6). Scale bar: 50 μm. From Ref. [33] with permission. (b) Low magnification, phase contrast micrograph of rabbit myocytes cultured for 16 days. The myocytes were cultured in a serum-free medium on laminin pre-treated plates.

of the cells started to become slightly rounded in appearance. After 6 days (Day 6) in culture, cells remained rod-shaped and cross-striated, and the main change was that cell ends became progressively more rounded. Rounding at the cell ends may be due to ultrastructural reorganisation. In rat myocytes intercalated discs became internalised within 24 h of culture [11]. Other changes in morphology may be due to modifications of the cell cytoskeleton with adaptation to a 2D surface. Fig. 2b shows a population of rabbit myocytes after 16 days in culture. Some of the myocytes still had an elongated morphology. However, nearly all showed signs of spreading at the ends of the cells. Thin membranous pseudopodia developed and these projected out into the local environment. With increasing time the pseudopodia spread laterally and also started to appear at other positions on the cell. The process of attachment to laminin treated surfaces and then subsequent spreading may be similar in all species cultured in serum-free medium (e.g. rat [37], rabbit [33] and cat [50]). In general, more than 50% of myocytes plated out remained rod-shaped after 7 days in culture. However, this is highly dependent on the quality of the isolation and may vary considerably. Differences of animal species and culture conditions (e.g. concentration of attachment substrate [22] and supplements added to the culture medium [37]) may also be important.

High resolution transmission electron micrographs of a mid-section of rat myocytes showed that the ultrastructure was similar to healthy intact cardiac tissue after 3 days in culture [37]. Fig. 3a shows an electron micrograph of a section through a rabbit myocyte cultured for 4 days. The myofilament organisation was well maintained, with the sarcomeres in good alignment with each other and clearly defined. Throughout the sarcomeres were numerous glycogen granules, which suggested that the myocytes were metabolically stable [51]. There were densely packed mitochondria, and running longitudinally, several long lengths of SR were observed. T-tubules were seen which retained their association with the Z line. After 1 week in culture, fluorescent labelling of actin and myosin and transmission electron microscopy (TEM) showed that myofilament organisation was not as well preserved in rat [37] and rabbit myocytes [22], and myofilament atrophy was occurring. However, feline myocytes may be more resistant to myofibrillar atrophy [42]. Cooper et al. (1986) reported that feline myocytes showed little morphological sign of myofibrillar atrophy for nearly 2 weeks in culture. It appears that the contractile apparatus of feline myocytes responds more favourably to culture on 2D laminin surfaces than rat and rabbit myocytes, although reasons for any differences between species are not clear.

Changes to T-tubule density in culture have been investigated using membrane potential sensitive fluorescent indicators [33,49]. In acutely isolated (Day 0) myocytes a striated pattern of fluorescent labelling, which covered most of the cross-sectional area of the cell, was observed [33] (Fig. 3b). The striated pattern was due to labelling of T-tubules and was not observed in atrial cells (e.g. Ref. [52]), which lack a T-tubule system [53]. In cultured myocytes, the density of T-tubules declined with time in culture [33]. Results from a population of myocytes suggested that T-tubules vanished from the cell ends first, which were also the areas rounding up and becoming structurally altered in other ways. The reduction in numbers of T-tubules, in addition to a gradual decrease of cell size (2D surface area and cell depth [33]) may have resulted in net loss of membrane surface area, and was consistent with mean values of membrane capacitance (directly proportional to membrane surface area), which also declined with time in culture [33,54].

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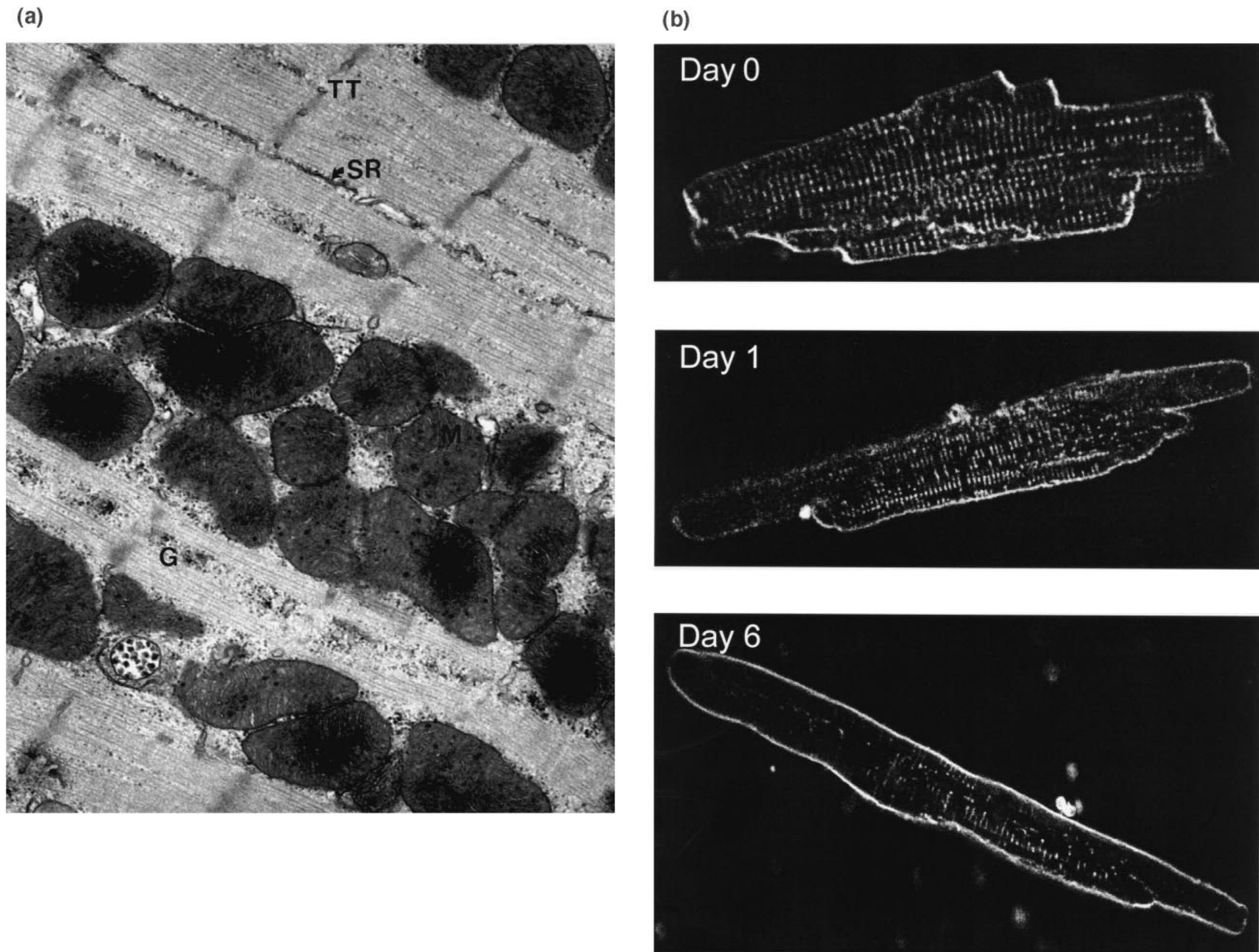

Fig. 3. (a) Transmission electron micrograph of the ultrastructural morphology of a rabbit myocyte cultured for 4 days in serum-free medium. Staining reveals well ordered myofilaments with large numbers of glycogen granules (G), mitochondria (M), sarcoplasmic reticulum (SR) and transverse tubules (TT). Magnification: ×28 512. (b) Myocytes after isolation (Day 0), after 24 h (Day 1) and after 6 days (Day 6) in culture, loaded with the membrane potential sensitive dye (di-4-ANEPPS; Molecular Probes) to image the transverse tubules (magnification ×40). Redrawn from Ref. [33] with permission.

3.2. Culture medium supplements

Early attempts to culture rat myocytes (e.g. Ref. [5]) used 5–15% serum to supplement culture medium. Although serum-supplemented medium permitted a high percentage of myocytes to remain viable in culture, gross changes in morphology occurred as the myocytes rounded up and de-differentiated. Piper et al. (1982) first successfully cultured rat myocytes in *serum-free* medium, and observed there were no gross changes in morphology over the first days of culture [32]. They demonstrated that myocyte separation and plating on an artificial 2D surface were not the only factors responsible for the dramatic changes in morphology observed in earlier studies. Their results suggested that in rat myocytes serum might fundamentally alter normal in-vivo properties and actively promote de-differentiation. Despite the advantage of serum-free medium, a major drawback for many applications was low survival rates with time in culture. Only 50% of myocytes plated out remained rod-shaped after 2 days in culture.

Survival of myocytes in serum-free culture was dramatically improved by supplementing the culture medium [37]. The basic medium (Medium 199 with antibiotics and 10^{-8} M insulin) was modified by adding creatine, L-carnitine and taurine and leaving out glutamine (CCT medium) [37]. At least when assessed by morphology, each change to the medium was additive. With all medium modifications combined, the longevity of rod-shaped myocytes increased to 50% remaining after 14 days in culture [37]. However, even in CCT medium, loss of protein mass still occurred. Volz et al. (1991) suggested this might be due to the absence of contractile activity and humoral factors, resulting in myofilament atrophy [37]. Consistent with this is the study of Ellingsen et al. (1993) [24] on action potential (AP) and contractile properties of rat myocytes, cultured in CCT medium plus additional supplements of 2 mg ml^{-1} bovine serum albumin and 3,5,3′triiodothyronine (reported

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to maintain the in-vivo ratio of α and β myosin heavy chains [55]). Contractile parameters became decreased by 30–50% in culture [24], perhaps as a result of myofilament atrophy. However the changes in contractile function might also have been due to: (1) Reduction in numbers of T-tubules. Loss of the T-tubule network has been demonstrated to cause marked spatial inhomogeneities of intracellular Ca (Ca_i) transients in guinea-pig myocytes, resulting from less reliable coupling between sarcolemmal Ca entry and SR Ca release [49]. A disruption of SR release might be expected to reduce contraction. (2) Changes in electrophysiological properties. Fig. 4(a,b) (from the work of Ellingsen et al. (1993) [24]) show that after 24 h in culture, AP duration (APD) is markedly reduced. This would be expected to reduce Ca entry during the AP and therefore decrease contractile properties. Paradoxically, the L-type Ca current density ($I_{Ca,L}$, normalised to cell size) increased significantly after 24 and 48 h in culture (Fig. 4c and d), which would normally be expected to increase APD. Therefore, presumably overlapping currents which favour early repolarisation of the AP may also have been

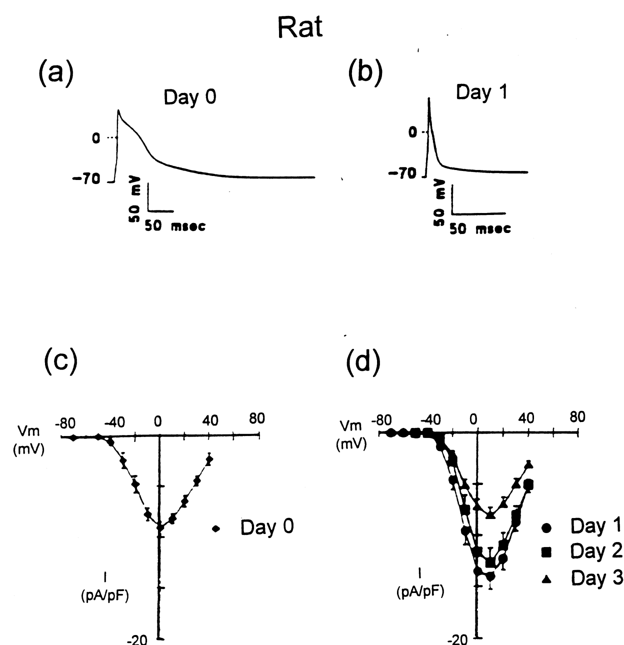

Fig. 4. Electrophysiological properties of adult rat ventricular myocytes cultured in Medium 199 with Earle's salts (without glutamine) supplemented with 25 mM HEPES and $NaHCO_3$, 2 mg ml^{-1} bovine serum albumin, 2 mM L-carnitine, 5 mM taurine, 5 mM creatine, 0.1 μM insulin and 10^{-10} M 3,5,3″-triiodothyronine. Action potential recordings from freshly dissociated (a) and Day 1 cultured (b) myocytes were obtained using whole cell current-clamp methods. The myocytes were superfused with Tyrode's solution and dialysed with a K-aspartate based intracellular solution. $I_{Ca,L}$ was recorded with the whole cell voltage-clamp technique, with step depolarisations (10 mV increments) from a holding potential of −80 mV. The external superfusate was Na and K free, and cells were dialysed with a Cs-aspartate solution plus 10 mM EGTA to buffer Ca_i to low levels. $I_{Ca,L}$-densities (mean±S.E.M.) are shown for freshly dissociated (Day 0, c) and cultured (d) myocytes. All experiments were performed at room temperature. Data from Ref. [24] with permission.

modulated in cultured rat myocytes. These currents include outward potassium (K) currents (for reviews see Refs. [56,57]) such as the rat delayed rectifier K current (I_K), ultra-rapid components of I_K (I_{Kur}) and transient outward K current (I_{TO}) which may have increased in culture. Alternatively a reduction of inward Na–Ca exchange current (which may also contribute to the AP plateau [58]) may have occurred. These currents have not yet been characterised in cultured rat myocytes. The effect of culture on several membrane currents and other electrophysiological properties is described in Section 3.3 and summarised in Table 1.

3.3. Electrophysiological properties

3.3.1. Resting potentials (RP) and APs

Electrophysiological properties have been studied in acutely isolated myocytes since the early 1980s [59,60]. Unlike intact tissue, it is possible in single myocytes to have adequate control of membrane potential (using microelectrode or patch-pipette voltage-clamp techniques) and in addition measure changes in intracellular ion concentrations using fluorescent indicators [36,61,62].

The study of Ellingsen et al. (1993) [24] demonstrates that measuring contractile properties of externally stimulated myocytes may not be a reliable method of assessing myocytes in culture. Since contraction and excitability of ventricular myocytes are influenced by AP shape and RP, it is important to also study these properties with time in culture. In addition, RP and AP shape are determined by underlying membrane currents, and so changes in RP and AP shape with time in culture reflect modifications of specific currents.

Fig. 5 shows APs recorded from feline (a) and rabbit (b) myocytes. The AP from Day 0 myocytes (Fig. 5ai) was characterised by a rapid upstroke, a ''prominent notch'' just after the upstroke, a plateau phase at potentials between +40 and 0 mV, and a rapid repolarisation after the plateau [14]. Mean RP for Day 0 feline myocytes was −78±0.5 mV. After 14 days in culture, the feline myocytes (which were maintained in medium supplemented with 5% foetal bovine serum and 5% NU serum, a synthetic serum substitute; Collaborative Research), had spread extensively, with only a central region remaining elongated and containing intact myofibrils [14]. The principal electrophysiological differences (Fig. 5aii) were a relatively depolarised RP (−71.9±0.7 mV), a reduced notch, and an increased APD (measured from upstroke to 90% repolarisation; APD_{90}). Some myocytes also exhibited early after depolarisations which were not observed in Day 0 myocytes [14].

The changes in rabbit RPs and APs with time in culture were more profound (Fig. 5b, from Ref. [33]). The rabbit myocytes were maintained in serum-free medium with no supplements to the medium (other than antibiotics) and recordings were made only from rod-shaped myocytes.

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Table 1
Overview of modulation of ionic currents in cultured myocytes

	Species and tissue, time in culture	First author, year and citation	Culture conditions		Morphology	Mean electrophysiological properties			
			Medium supplements	Attachment substrate		Current amplitude	Current density	Activation	Inactivation
Sodium current (I_{Na})	Human atria: 5 days	Feng, 1996 [65]	10% FBS	None	Spherical	↓	No change	No change	No change
	Feline ventricle: 9–14 days	Schackow, 1995 [121]	5% FBS, 5% NU-S & ITS	Laminin	Spread	–	↑	Shift −13 mV	Shift −20 mV
	As above	As above	5% FBS, 5% NU-S & ITS	3D-Alginate matrix	Rod-shape	–	↓	Shift −18 mV	Shift −16 mV
L-type calcium current ($I_{Ca,L}$)	Rabbit ventricle: 6 days	Mitcheson, 1996 [33]	None	Laminin	Rod-shape	↓	No change	No change	No change
	Rabbit ventricle: 4 days	Mitcheson, 1997 [54]	T, Cr, C, I	Laminin	Rod-shape	↓	No change	No change	No change
	Feline ventricle: 9–14 days	Schackow, 1995 [14]	5% FBS, 5% NU-S & ITS	Laminin	Spread	–	↑	No change	–
	As above	As above	5% FBS, 5% NU-S & ITS	3D-Alginate matrix	Rod-shape	–	↓	Shift −6 mV	–
	Rat ventricle: 1–3 days	Ellingsen, 1993 [24]	A, C, Cr, I, T, T_3	Laminin	Rod-shape	–	↑ (Day 1–2) No change (Day 3)	–	No change
	Rat ventricle: 2 days	Berger, 1994 [13]	A, C, Cr, I, T, T_3	Laminin	Rod-shape	–	↑	–	No change
	Human atria: 14 days	Benardeau, 1997 [26]	10% FCS, I	None	Spread	↑	No change	No change	No change

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Table 1. Contd.

	Species and tissue, time in culture	First author, year and citation	Culture conditions		Morphology	Mean electrophysiological properties			
			Medium supplements	Attachment substrate		Current amplitude	Current density	Activation	Inactivation
Transient outward potassium current (I_{TO})	Rabbit ventricle: 6 days	Mitcheson, 1996 [33]	None	Laminin	Rod-shape	↓	↓	–	No change
	Feline ventricle: 9–14 days	Schackow, 1995 [14]	5% FBS, 5% NU-S & ITS	Laminin	Spread	↓	↓	No change	–
	As above	As above	5% FBS, 5% NU-S & ITS	3D-Alginate matrix	Rod-shape	↓	↓	No change	–
	Human atria: 5 days	Feng, 1996 [65]	10% FBS	None	Spherical	↓	↓	No change	Shift by −21 mV
	Human atria: 7 days	Benardeau, 1997 [26]	10% FCS, I	None	Spread	–	No change	–	–
Ultra-rapid activating potassium current (I_{Kur})	Human atria: 7 days	Benardeau, 1997 [26]	10% FCS, I	None	Spread	↑	↑	–	–
	Human atria: 5 days	Feng, 1996 [65]	10% FBS	None	Spherical	No change	↑	No change	–
Inward rectifier potassium current (I_{K1})	Rabbit ventricle: 6 days	Mitcheson, 1996 [33]	None	Laminin	Rod-shape	↓	↓	–	–
	Feline ventricle: 9–14 days	Schackow, 1995 [14]	5% FBS, 5% NU-S & ITS	Laminin	Spread	–	↓	No change	–
	As above	As above	5% FBS, 5% NU-S & ITS	3D-Alginate matrix	Rod-shape	–	↓	No change	–
	Human atria: 7 days	Benardeau, 1997 [26]	10% FCS, I	None	Spread	–	↓	–	–

Table key: FBS, foetal bovine serum; FCS, foetal calf serum; NU-S, NU serum; ITS, insulin transferrin selenium; A, albumin; C, L-carnitine; Cr, creatine; I, insulin; T, taurine; T_3, triiodothyronine; ↑, parameter increased; ↓, parameter decreased; –, parameter not measured.

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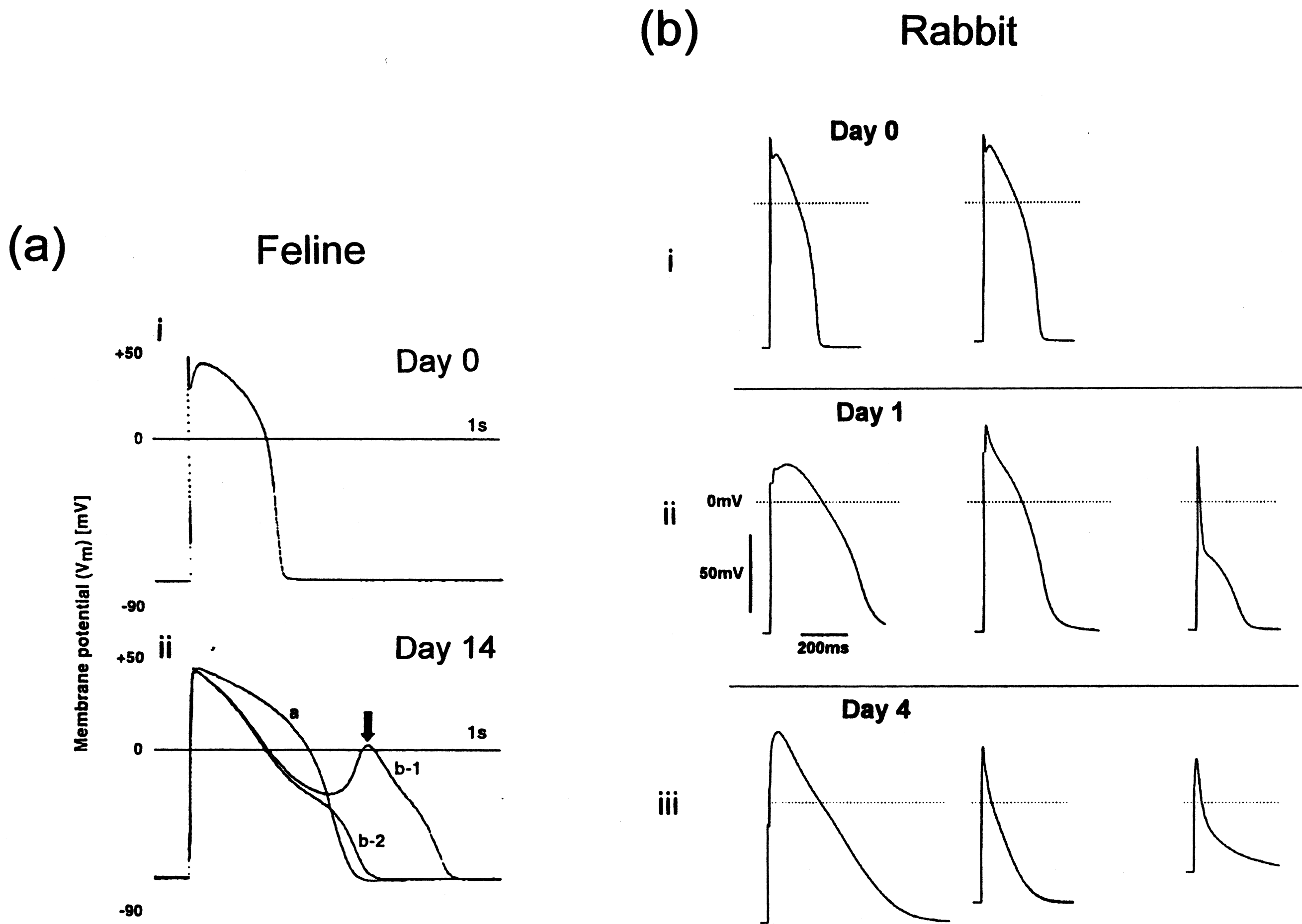

Fig. 5. Representative action potential (AP) recordings from feline (a) and rabbit (b) myocytes. (a) AP recordings from a feline Day 0 (i) and two cultured Day 14 (ii) myocytes. The Day 14 myocytes were cultured on laminin coated glass coverslips and cultured in Eagle's minimum essential medium (MEM) plus 25 mM HEPES, supplemented with 5% foetal bovine serum, 5% NU serum and ITS (2.5 μg ml^{-1} insulin, 2.5 μg ml^{-1} transferrin and 2.5 ng ml^{-1} selenious acid). APs were recorded at room temperature in a physiological saline external solution, a K-glutamine based intracellular solution and elicited using an intracellularly applied stimulus. b-1 And b-2 are two consecutive action potentials from the same cell, with a 5 s interval. In three out of eight Day 14 cultured myocytes, early after depolarisations (EADs, e.g., b-1) were observed. From Ref. [14] with permission. (b) APs from Day 0 (i), Day 1 (ii) and Day 4 (iii) rabbit myocytes. The rabbit myocytes were cultured on laminin pre-treated glass coverslips and maintained in Medium 199 with no added supplements other than 25 mM Na bicarbonate and antibiotics. APs were recorded in Tyrode's solution (at 37°C) and stimulated via a glass patch-pipette filled with a KCl based solution. From Ref. [33] with permission.

The AP of Day 0 rabbit cells (Fig. 5bi) was similar to Day 0 feline myocytes, and variation in AP shape between cells was small. By comparison, there was a large variation in APs recorded from Day 1 and Day 4 cells (Fig. 5bii and 5biii). In Day 1 myocytes the mean RP was similar to Day 0 cells, however the plateau phase varied substantially between cells [33]. By Day 4 there was increased RP variability and the overshoot potential was significantly reduced. Applying negative holding current to artificially hyperpolarise the RP to −80 mV caused overshoot potentials to increase, suggesting that Na channels were not irreversibly lost, but may have been partially inactivated.

3.3.2. Membrane currents in cultured myocytes

Despite the changes in AP shape between species, there may nevertheless be a number of trends:

1. RPs in cultured myocytes appeared less negative, and less stable than in Day 0 myocytes [14,33]. In Day 0 myocytes the RP is close to E_K (reversal potential for K ions). A depolarised RP in culture might be due to a decreased membrane permeability to K (relative to other inward conductances). Consistent with this, inward rectifier K current (I_{K1}), recorded using whole cell voltage-clamp in rabbit myocytes, declined progressively with time in culture [33] (Fig. 6a). In feline myocytes cultured on laminin, I_{K1} normalised to membrane capacitance (to compensate for variation in cell size between cells) was reduced by greater than 30% [14]. It seems likely, therefore, that either the number of active I_{K1} channels decreased with time in culture or modulation of I_{K1} channels occurred.
2. The small early notch in the AP plateau in Day 0 myocytes was rarely observed in the APs of cultured

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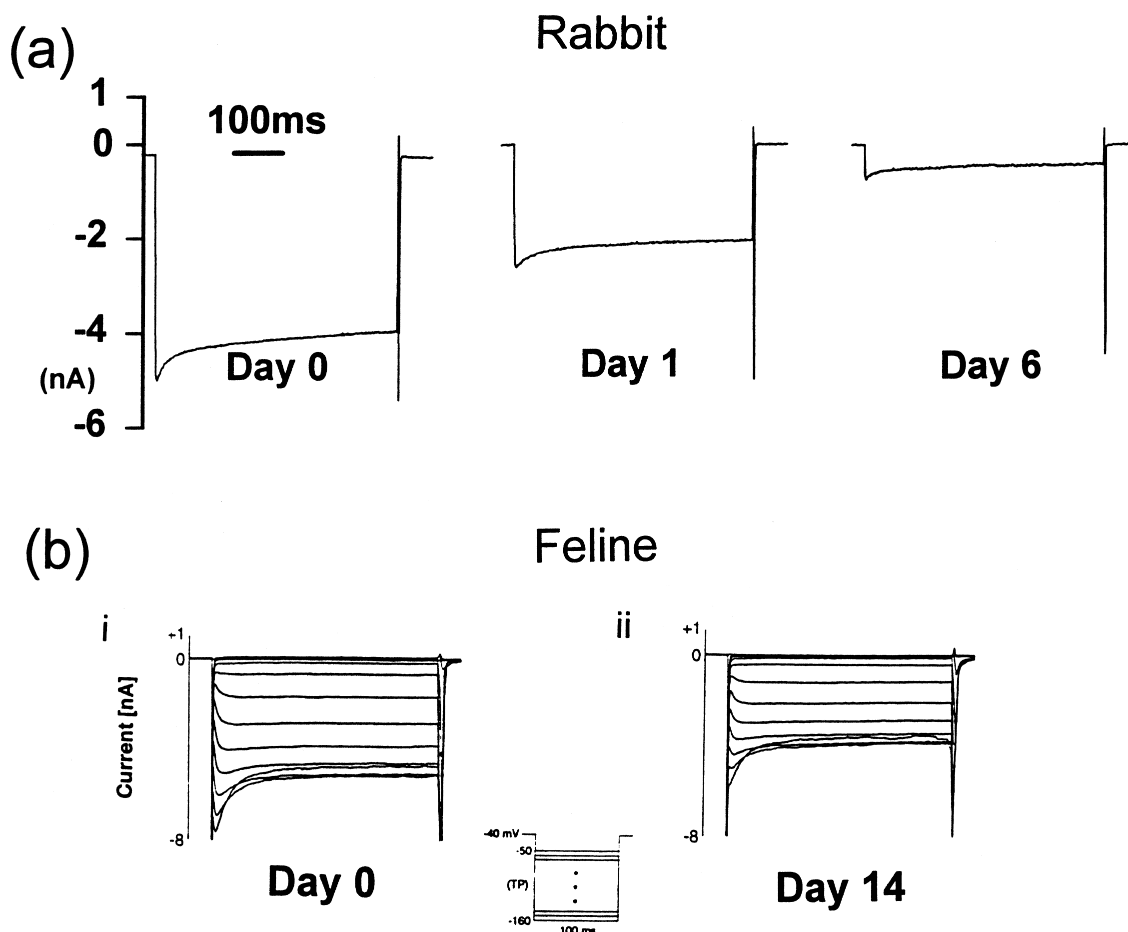

Fig. 6. (a) Whole cell voltage-clamp recordings of inward rectifier K current (I_{K1}) from Day 0, Day 1 and Day 6 rabbit myocytes (see Fig. 5b for culture conditions). Rabbit cells were voltage-clamped at a holding potential of −40 mV and 500 ms voltage pulses applied to a test potential of −130 mV. A KCl based intracellular solution with 5 mm BAPTA was used and the cells superfused in Tyrode's solution at 37°C. Data from Ref. [33]. (b) Whole cell voltage-clamp recordings of I_{K1} from feline Day 0 (i) and cultured Day 14 (ii) myocytes (see Fig. 5a for culture conditions and recording solutions). Feline myocytes were voltage-clamped at a holding potential of −40 mV and currents elicited by a series of 100 ms hyperpolarising test pulses from −50 to −160 mV in 10 mV increments (b, inset). From Ref. [14] with permission.

rabbit and feline myocytes. The small notch is known to be due to I_{TO} (e.g. Ref. [63]). Fig. 7a shows typical recordings of I_{TO} in cultured rabbit myocytes. I_{TO} amplitude decreased progressively with time in culture [33]. No significant changes in current–voltage relations or steady-state inactivation kinetics were observed. In feline myocytes cultured on laminin for 14 days, I_{TO} was substantially smaller (Fig. 7b), whilst the voltage-dependent properties of the remaining I_{TO} were also unchanged [14]. A preliminary study reported no differences in I_{TO} with time in culture in rat myocytes [24], but data to support this has not yet been published.

$I_{Ca,L}$ is a characteristic feature of heart cells, and has been investigated in a number of culture studies. As discussed previously, peak $I_{Ca,L}$-density in rat myocytes cultured in supplemented medium increased by 55% after 24 h in culture (see Fig. 4c,d) and was still larger than in Day 0 cells after 48 h, before returning to control levels after 72 h [24]. $I_{Ca,L}$ recorded from feline myocytes after 14 days in culture was also significantly larger than $I_{Ca,L}$ measured in Day 0 feline myocytes (Fig. 8a; [14]). However, in this study, $I_{Ca,L}$ was not measured selectively. Therefore part of the apparent increase of inward current might result from a decrease of I_{TO} (as described previously) or other overlapping currents in culture, rather than an actual increase in $I_{Ca,L}$.

3.3.3. Supplemented versus non-supplemented medium

L-type Ca channel activity has been measured selectively in rabbit myocytes as Ba current ($I_{Ba,L}$; see Fig. 8 legend) [33,54]. To determine if modifying the culture medium was able to alter membrane currents in culture, $I_{Ba,L}$ was recorded in myocytes maintained in non-supplemented and supplemented (CCT) medium. In supplemented medium, $I_{Ba,L}$ absolute amplitude (Fig. 8bi) declined with a similar time course to capacitance (resulting from concomitant decreases of cell size and T-tubule density). Therefore, $I_{Ba,L}$-density (Fig. 8bii) in myocytes cultured in supplemented medium, did *not*

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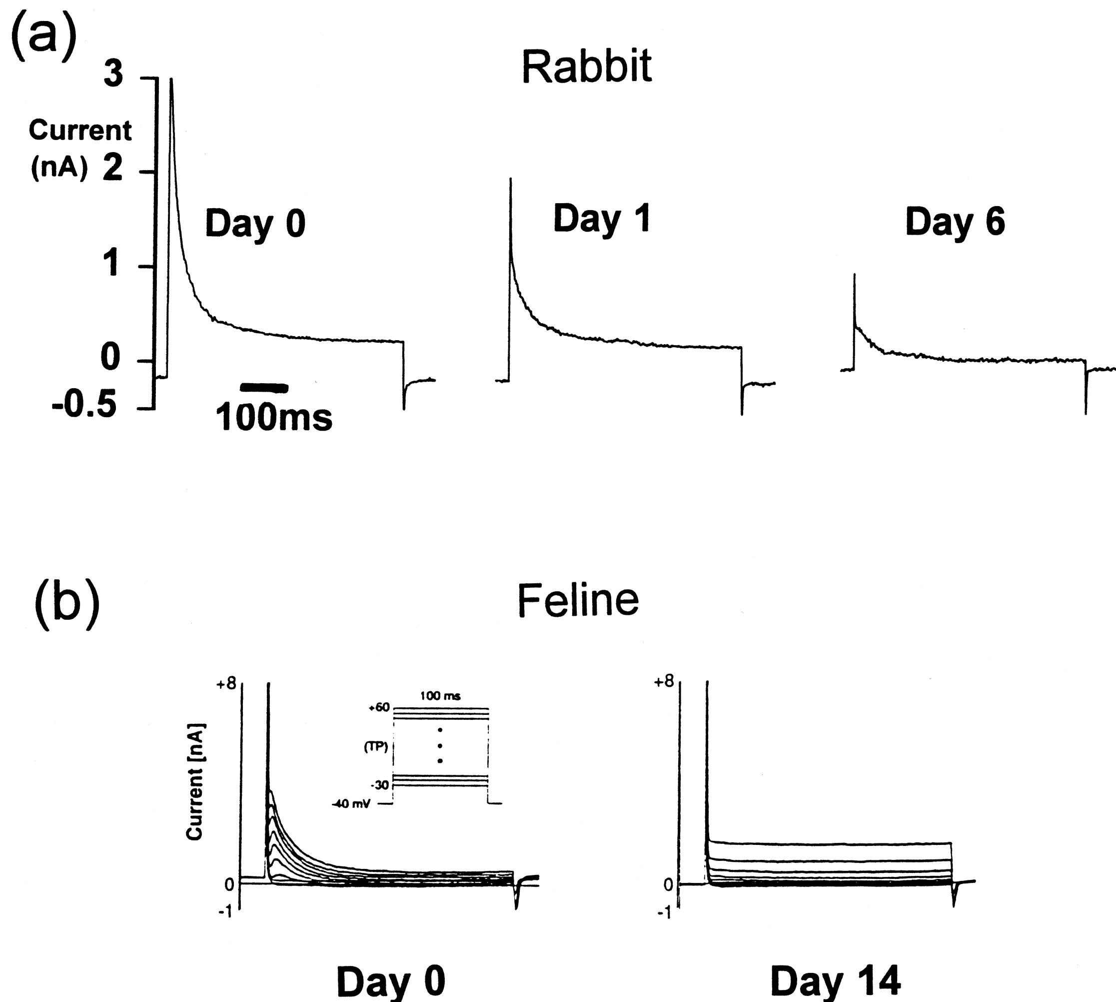

Fig. 7. (a) Recordings of transient outward K current (I_{TO}) from Day 0 and cultured rabbit myocytes (Day 1, Day 6; culture conditions as in Fig. 5b). The myocytes were superfused with a Na-free solution (to eliminate I_{Na}) containing 20 μM nifedipine to inhibit $I_{Ca,L}$ and dialysed with a KCl based intracellular solution containing 5 mM BAPTA. The rabbit myocytes were voltage-clamped at a holding potential of −80mV and test potentials applied to +60 mV for 500 ms. Data from Ref. [33]. (b) Recordings of I_{TO} from feline Day 0 and Day 14 cultured myocytes (culture conditions and recording solutions same as Fig. 5a, except that 0.5 mM $CdCl_2$ was applied to inhibit $I_{Ca,L}$). Feline myocytes were voltage-clamped and membrane potential stepped to a series of depolarising pulses (−30 mV to +60 mV in 10 mV increments for 100 ms; inset) from a holding potential of −40 mV. From Ref. [14] with permission.

change significantly with time [54]. In marked contrast, $I_{Ba,L}$-density in myocytes maintained in non-supplemented medium changed substantially during culture, suggesting that simple changes to the culture conditions may alter preservation of membrane currents.

Measurements of $I_{Ca,L}$ in rat, feline and rabbit myocytes may vary because of differences in specific culture conditions employed or because of species-dependent adaptation to culture. Two recent studies have been published on myocytes isolated from human right atrial appendage [64,65], which suggest that culture conditions are critical in modulating expression patterns of some ion channels. In both studies myocytes were cultured in serum-supplemented medium and plated without attachment substrates. Despite the similarity of the cultures, significant differences in the properties of ionic currents were reported. Hatem et al. (1996) observed a serum-induced growth resulting in a doubling of membrane capacitance between acutely isolated and 8 day old cultured myocytes [64]. They found that the current density of I_{Kur} (ultra-rapidly activating, non-inactivating potassium current, also known as I_{SUS}), increased dramatically in culture, but I_{TO}-density did not change. In contrast, Feng et al. (1996) reported that I_{Kur}-densities increased only moderately with time in culture, due to a *decrease* in cell size, and I_{TO}-density declined [65]. The differences of I_{Kur} and I_{TO} between studies were interesting because they could only be attributed to unidentified, batch-dependent serum factors. An interesting question is whether the differences of membrane currents were an indirect effect on cell growth, induced by the serum factors. There are several reports of I_{TO} amplitude decreases associated with hypertrophy in rat

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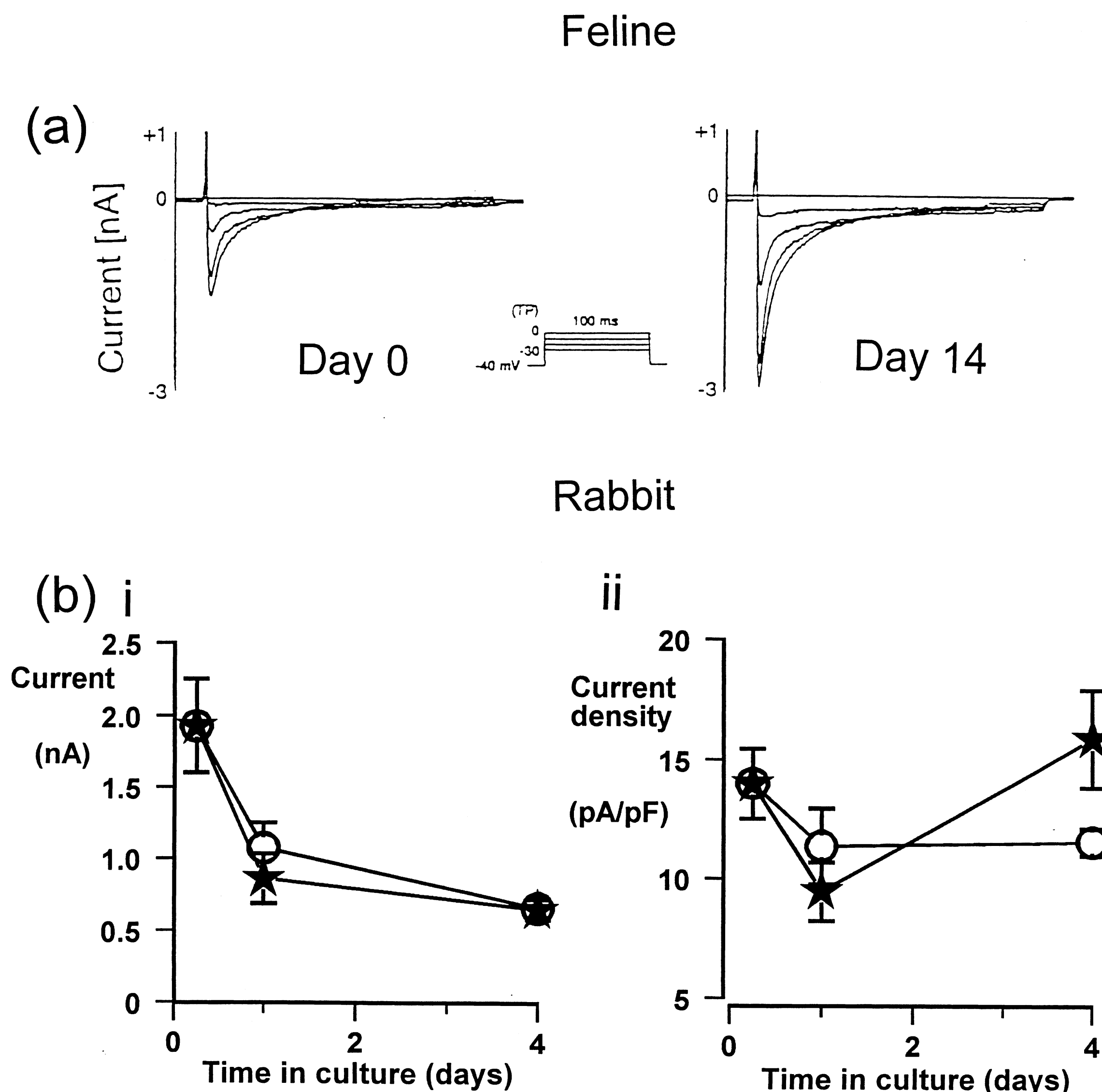

Fig. 8. (a) Families of L-type Ca currents ($I_{Ca,L}$) recorded from feline Day 0 (left panel) and Day 14 cultured myocytes (right panel, culture conditions and recording solutions same as in Fig. 5a). $I_{Ca,L}$ was elicited by applying 100 ms depolarising voltage pulses to membrane potentials between −30 and 0 mV (see inset) from a holding potential of −40 mV. Data from Ref. [14] with permission. (b) Mean $I_{Ba,L}$ absolute amplitudes (i) and $I_{Ba,L}$-densities (ii) from rabbit Day 0 and cultured myocytes. Current through L-type Ca channels was measured, substituting external Ca with Ba, so that $I_{Ca,L}$ was measured as Ba entry through the Ca channel ($I_{Ba,L}$), preventing Ca_i induced inactivation of the Ca channel. Membrane potential was stepped to +10 mV from a holding potential of −80 mV in voltage-clamped myocytes. (★) Results from myocytes cultured in Medium 199 with no supplements other than antibiotics. (○) Results from myocytes cultured in Medium 199 supplemented with (in mM) 5 creatine, 5 taurine, 2 L-carnitine, 2.5 pyruvate, and 10^{-7} M insulin. Data are presented as means±S.E.M.. Redrawn from Ref. [54] with permission.

myocytes, which may be in part responsible for changes in AP shape [66,67]. The in-vitro study of Hatem et al. (1996) may provide evidence that cell growth is associated with changes of I_{TO} amplitude in human myocytes, although the precise mechanism of modulation remains unclear.

3.3.4. Differential effects of culture on ion channels

One conclusion of experiments on cultured myocytes is that different ion channel expressions may be regulated independently of one another, and by separate mechanisms [64,65]. In the study of Hatem et al. (1996), addition of 5–10 nM staurosporine (to inhibit protein kinase C) reduced I_{TO}-density but not the increase of I_{Kur}-density with culture. Feng et al. (1996) showed that I_{Kur}, I_{TO} and I_{Na} were each modulated in different ways. Interestingly, whereas activation properties of I_{TO} remained constant, inactivation properties (recovery, voltage- and time-dependent) were altered with time in culture. There is increasing evidence that *Shal*-like potassium channel subunits (Kv4.x), rather than Kv1.4 may be predominantly responsible for I_{TO} channels in cardiac tissue [68]. However, it remains uncertain whether the changes of I_{TO} inactivation in culture might result from either (1) a change in

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composition of Kv subunit heterotetramers, perhaps resulting in a net loss of proteins with N-type inactivation [56,57], or (2) modulation by β-subunits, which alter the inactivation properties of cloned potassium channel subunits [69], and have been identified in human atrial tissue. Antisense DNA strategies against specific Kv subunits have been performed using cultured myocytes, to identify the Kv subunits responsible for formation of functional K channels, and these will be described in Section 3.7.

Possible mechanisms which might account for differential ion channel modulation in culture include:

1. *Spatial location of different ion channels in the membrane*. Channels located predominantly on regions of the cell which rapidly change during culture (such as cell ends), may be lost more quickly than channels located in more stable areas of the cell (such as the surface membrane in central regions). This may be particularly important for channels which are anchored (or even modulated) by the cell cytoskeleton, which is likely to be in a dynamic state as the myocytes adapt from a 3D to a 2D environment.
2. *Regulation of ion channel expression and turn-over*. The number of functional channels expressed in the membrane may depend on the balance between the number of channels being produced and inserted versus rate of channel degradation. It seems unlikely that the same factors will control both expression and breakdown of ion channels. In cultures in which the medium is supplemented by serum, an unknown number of active substances may be exerting an effect on cell biological processes such as transcription, translation and organisation of intracellular membrane traffic, all of which might be expected to modulate ion channel expression.
3. *Alteration of cellular mechanisms that modulate channel activity*. The kinetic properties of channels can be modulated by phosphorylation pathways and association with regulatory proteins (e.g. GTP-binding proteins) [70]. If these regulatory pathways change in culture, there may be concomitant effects on channel activity.

3.4. Continuous electrical stimulation of contraction during culture

Myocytes cultured using the rapid attachment method are quiescent. Repetitive and regular depolarisation and contraction is absent, and may contribute to observed myocyte atrophy. Depolarisation, elevated cytosolic Ca and contraction may be important stimuli for regulation of many cell functions, including expression of contractile proteins and membrane ion channels [71].

Kato et al. (1995) studied effects on feline myocyte morphology and protein synthesis of electrically stimulating contraction during culture [50]. Myocytes were attached to laminin pre-treated culture dishes and maintained in serum-free medium. Contraction was stimulated by 5 ms duration voltage-pulses through carbon electrodes (frequency 1 Hz). The protein content of non-stimulated rod-shaped myocytes decreased by 16% between Days 1 and 4 of culture and then remained constant up to Day 7. However protein content of continually paced myocytes, remained constant between Days 1 and 4 and *increased* between Days 4 and 7 (Fig. 9a). Rate of protein synthesis during culture was similar to the change of protein content, suggesting that alteration of protein synthesis (and not degradation) was responsible.

Berger et al. (1994) studied rat myocytes stimulated at frequencies of 0.1 to 5 Hz [13]. There was an initial decrease in contractile properties (measured as peak displacement, contraction and relaxation velocity; Fig. 9b–d) after 6 h, which was then maintained over the next 36 h. After this, contractile function began to increase. After 72 h, values for contractile parameters were higher in paced cultured myocytes than acutely isolated myocytes. $I_{Ca,L}$-density was greater in paced, compared to quiescent cells, but no comparisons were made with acutely isolated myocytes. AP parameters, which might be responsible for changes in contractile function were also not measured. Nevertheless, the results suggest that continuous electrical pacing helps prevent the decline of contractile properties typical of quiescent myocytes [24]. Inhibiting contraction of paced cultured rat myocytes by addition of verapamil to the medium resulted in reduction of contractile function (measured once verapamil had been washed off) to the same levels as non-paced myocytes [13]. This suggested that *contraction*, rather than membrane depolarisation, maintained electromechanical function. Total protein content and rates of protein synthesis were significantly greater in paced than quiescent cultures [72], consistent with results from feline myocytes [50]. The results suggest that cell hypertrophy may occur because of an increase in work load, without any additional growth factors. These experiments would not have been as conclusive if the myocytes had been cultured in serum-supplemented medium, since exogenous hormones and growth factors are present in serum.

3.5. Culturing myocytes in 3D matrices

Another technique used successfully to maintain rod-shaped morphology of cultured myocytes is to embed freshly isolated cells in a 3D matrix [21]. In an interesting study by Decker et al. (1991) [21] adult feline myocytes were encased in polymerised calcium alginate beads and maintained in medium supplemented with 5% FCS and 5% chemically defined serum substitutes. After 2 weeks, myocytes from the same isolation, but cultured on a 2D surface had flattened and spread extensively [21]. In contrast myocytes in the 3D matrix remained rod-shaped

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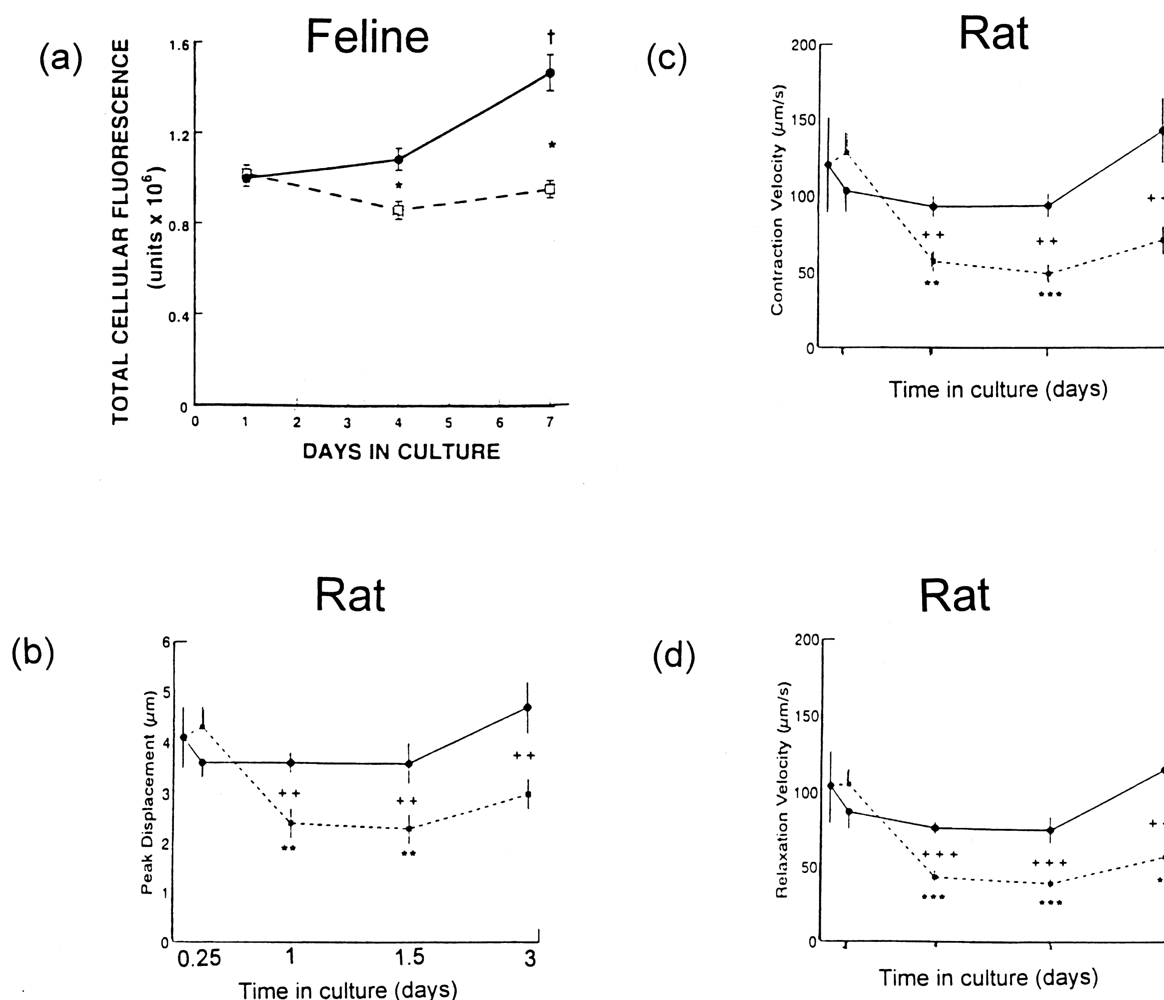

Fig. 9. The effects of repetitive electrically stimulated contraction on cultured myocytes. (a) Protein content per cell in quiescent (□) and electrically stimulated (●) feline myocytes. Feline myocytes were plated on laminin pre-treated culture dishes and maintained in serum-free Medium 199 supplemented with 100 μM ascorbic acid, 0.2% (w/v) bovine serum albumin, 5 mm creatine, 5 mm taurine, 2 mM L-carnitine, 10 μM cytosine arabinoside and 10^{-7} M insulin. Total protein for individual myocytes was determined by staining with fluorescence-tagged isoithiocyanate (FITC) and cellular fluorescence measured with a confocal microscope. * $P<0.05$ compared to quiescent. † $P<0.05$ compared to Day 1 stimulated cells. Values are means±S.E.M. Data from Ref. [50]. (b–d) Contractile properties of quiescent (dashed line) and continually stimulated (solid line) rat myocytes. Rat myocytes were cultured on laminin pre-treated dishes and maintained in serum-free Medium 199 supplemented with 2% bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 1.3 mM L-glutamine, 10^{-7} M insulin, 10^{-10} M triiodothronine, 2.5 mM pyruvate. Data are shown for absolute amplitude of contraction (b), velocities of shortening (c), and velocities of relaxation (d). ** $P<0.05$; *** $P<0.001$ compared with Day 0 myocytes; and $^{++}$ $P<0.05$; $^{+++}$ $P<0.01$ paced versus quiescent cells. Data from Ref. [13] with permission.

(despite being in a serum-containing medium) [21]. Both preparations were quiescent and so contractile activity was not a variable in this study. With TEM, ultrastructural morphology at the core of individual myocytes in the 3D cultures was well preserved. However, they did notice a region under the sarcolemma which did not contain myofilaments. This region was organelle rich, but no attachments between the sarcolemma and the myofibrillar apparatus were observed. No mechanical points of attachment were observed between the sarcolemma and the alginate matrix either. In addition, when the fractional cell volume taken up by the myofilaments was quantified, it was found to be significantly decreased compared to acutely isolated myocytes [21]. These results appear consistent with mechanical load on the myocyte myofibrillar apparatus playing a role in preventing atrophy. However, in 2D cultures, mechanical load on myofilaments was reported to be preserved by attachment to laminin [42] and yet greater disruption of myofilament organisation was observed. These results may suggest that structural alterations and myofibrillar atrophy are due to both modifications of cell shape as adaptation to 2D surfaces occurs, and to changes in mechanical load on quiescent myocytes.

Electrophysiological recordings from feline myocytes cultured in either 3D and 2D conditions allowed assessment of whether changes were a result of cell shape, or due to culture per se [14]. APs, I_{K1}, I_{TO} and $I_{Ca,L}$ in acutely isolated myocytes were compared with 9–14 day

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myocytes, cultured on either 2D laminin pre-treated surfaces or embedded in 3D alginate matrices. Decreases of I_{TO} and peak I_{K1} occurred in myocytes from both 2D and 3D cultures, suggesting that the decline in amplitude of these currents was independent of culture-induced morphology changes. However, APD_{50}, $I_{Ca,L}$ amplitude and number of incidences of early after depolarisations were significantly increased in myocytes from 2D cultures, and not in myocytes from 3D cultures. These results provide further evidence that channel expression and channel turnover may be regulated by distinct mechanisms which are sensitive to culture in different ways.

3.6. The role of mechanical load

The 3D cultures described in Section 3.5 have well preserved ultrastructural morphology. Nevertheless, regions beneath the sarcolemma appeared to become modified, and myofibrillar atrophy occurred. No mechanical interactions were observed between the myocyte and the alginate matrix, suggesting that the myocytes were simply ‘‘trapped’’ in the matrix, without any mechanical load on the cytoskeleton and contractile apparatus. Feline myocytes externally loaded by adhesion to laminin substrates retained the structural, biochemical and functional properties of fully differentiated myocytes for over 2 weeks in culture in serum-free medium [42]. In contrast, myocytes which were otherwise treated in exactly the same way, but were unattached and therefore externally unloaded rapidly lost differentiated characteristics, and began to resemble neonatal myocytes [42], suggesting that substrate adhesion alone was important for maintaining differentiation. Increasing the load on single myocytes by stretching the surface the myocytes were attached to and increasing resting length, resulted in increased mRNA and protein synthesis activity [73]. Furthermore, electrically stimulating contraction in acutely isolated myocytes in order to actively increase mechanical load, accelerated protein synthesis compared to quiescent myocytes [74]. Two other studies were consistent with the possible role of contraction modulating morphology and functional properties of myocytes in culture. (1) β-Adrenergic stimulation of contraction was reported to preserve ultrastructural morphology and promote cell growth [75]. Applying nifedipine to block contraction of β-adrenergic stimulated cells resulted in significant myofibrillar atrophy and decreased density of myosin staining [75]. (2) In high density cultures of rabbit myocytes, synchronous spontaneous contractions occurred which resulted in maintenance of myofibrillar structure [23]. In quiescent, lower density cultures myofibrillar organisation was not as well preserved.

3.7. Future directions for cultured myocytes using molecular biological techniques

At present it is not possible to maintain all the properties of cultured myocytes identical to acutely isolated cells, however for many applications this is not critical. If one or more aspects of cardiac physiology remain unchanged in culture, they can be investigated using molecular biological techniques, irrespective of changes in other unrelated properties. A recent study of the molecular basis of I_{Kur} demonstrated this point [16]. As described earlier, Feng et al. (1996) found that although changes of I_{Kur}-density occurred in culture, all other properties of I_{Kur} remained unaltered [65]. They could then investigate the molecular basis of I_{Kur} by using antisense oligodeoxynucleotides directed against Kv1.5 mRNA [16]. Anti-Kv1.5 oligonucleotides specifically decreased the amplitude of I_{Kur} compared to control currents (Fig. 10), but the properties of I_{TO} were unaltered. They were able to conclude that Kv1.5 channel subunits were essential to the expression of I_{Kur}, but not I_{TO}, in human atrial cells, and

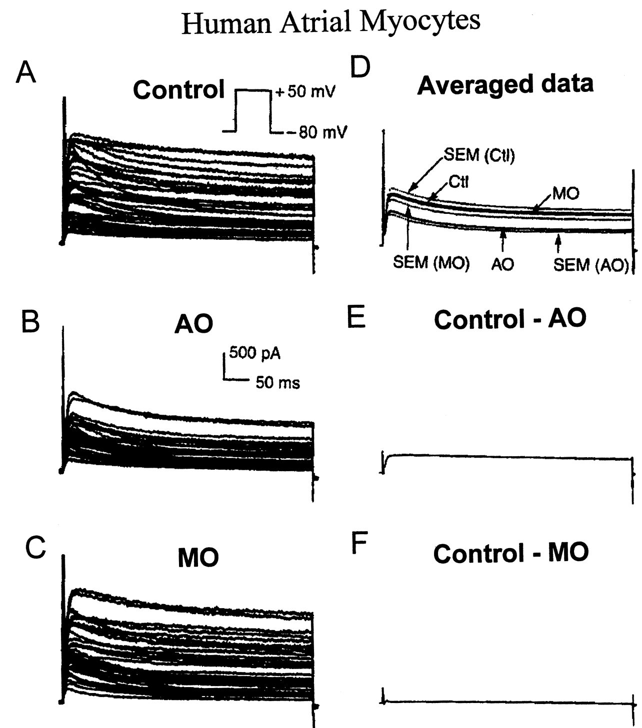

Fig. 10. Effects of antisense (AO) or mismatch (MO) oligonucleotides on membrane currents recorded from cultured human atrial myocytes maintained in Medium 199 supplemented with 10% serum. AO or MO (5 μmol l^{-1}) treatment was started 24 h after plating and continued for 48 h, after which membrane currents were recorded upon depolarisation from a −80 mV holding potential to test potentials of +50 mV for 500 ms. Cells were superfused with a 0 Na-based extracellular solution (to eliminate I_{Na}) containing 200 μM $CdCl_2$ to inhibit $I_{Ca,L}$. The intracellular solution was K-aspartate based. (A–C) Each panel shows 39 recordings selected randomly from 55 cells exposed to control medium (A; oligonucleotide vehicle only), AO (B), or MO (C). (D) Averaged currents from all 55 cells from each group. (E) Membrane currents obtained by digital subtraction of averaged currents of AO treated cells from averaged currents from control cells. (F) Currents obtained after subtraction of averaged current from MO treated cells from averaged currents from control cells. Redrawn from Ref. [16] with permission.

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thereby provided direct evidence for the role of a cloned K channel subunit in a macroscopic cardiac K channel.

Other ways of investigating protein function in the cardiovascular system include screening for mutations in zebrafish [76–78]. The heart and blood vessels are easily visualised in the transparent zebrafish embryo [79], and mutations that effect morphogenesis [80] or function [81] can be identified. Transgenic techniques provide the opportunity to target over-expression of gene products, or selectively knock out genes, in specific tissues and cell types (for recent reviews see Refs. [82–85]). The effect of manipulating expression of specific genes can be investigated at the whole animal and cellular level. To assess the physiological and pathological consequences of transgene expression, the normal physiological properties of small mammalian models, particularly the mouse, need characterising [86,87]. Considerable information about the physiological properties of rat embryonic/neonatal and adult cardiac myocytes already exist (e.g. Refs. [6,88–95]), and information is now also becoming available for mice [87,96–102]. Additional techniques for manipulating protein expression are antisense oligonucleotide strategies (to block expression, e.g. Ref. [103]) or adenovirus techniques (e.g. Ref. [19], to over-express a selected protein). In both cases, time is required for the changes to occur, and this may be as long as 2 to 4 days. Acutely isolated myocytes do not remain viable for this long and so cultured cells are needed. Cardiac cell lines, displaying a variety of phenotypes [104–114], provide a replenishable supply of cells and may be suitable for many experiments. Both cell lines and neonatal myocytes can also be transfected with plasmids encoding exogenous genes relatively easily [103,115–117]. However, for the molecular dissection of mechanisms that exist in the adult heart in-vivo, use of *fully* differentiated adult cardiac myocytes is preferable.

There are now a number of methods for importing exogenous DNA into cells which might be effective for adult cardiac myocytes. Cationic liposomes (e.g. lipofectamine) can be used to permeabilise the cell membrane and allow plasmids or oligonucleotides to enter [103], although low efficiencies of transfection may be prohibitive for many studies. For highly efficient gene transfer, the innovative technique of infecting myocytes with recombinant adenovirus can be used [19]. Replication deficient strains of adenovirus were used as a vector for transferring exogenous β-galactosidase or luciferase DNA with a promoter region into cultured myocytes. Transfection efficiencies of more than 80% were possible without reduced cell viability after 2 days, and with only a small decrease in the percentage of rod-shaped myocytes compared to adenovirus-uninfected myocyte cultures [19]. Adenovirus-mediated gene transfer has been instrumental in investigating signalling pathways for transcriptional control in response to trophic external stimuli, and for repression of cardiac cell division [118,119]. The β-adrenergic signalling pathway has also been upregulated using adenoviral-mediated gene transfer [15]. Two proteins, the human β_2-adrenergic receptor and a peptide inhibitor of β-adrenergic receptor kinase (an enzyme that phosphorylates and uncouples agonist bound β-receptors), were over-expressed in cultured adult rabbit ventricular myocytes. In response to isoprenaline treatment, both transgenes were shown to increase levels of intracellular cAMP and adenyl cyclase activity [15].

An additional transfection technique is to package DNA into liposomes coated with ''ultraviolet light inactivated hemaglutinating virus of Japan'' (HVJ). HVJ is reported to augment fusion of the liposome with the surface membrane of targeted cells, resulting in delivery of the DNA into the cell with high efficiency [120]. Possible advantages of the technique are that unlike adenovirus gene transfer, DNA of any size can be packaged and existing constructs can be used without additional preparation. This technique has been shown to be effective on rat neonatal myocytes, it is not yet known if it will also be as effective with adult myocytes in culture, although in-vivo transfection of adult rabbit myocardium has been demonstrated [120].

4. Summary

In Sections 1 and 2 of this review, the relative merits and problems of cultured myocytes were presented, and a detailed method for isolating and culturing adult myocytes described. Manipulation of the culture environment has resulted in improved preservation of the normal physiological properties of adult myocytes in vitro. Continuous electrical stimulation in culture, and maintaining myocytes in a 3D matrix, are relatively new and promising techniques. More work is needed to evaluate preservation in the long term, particularly when used in combination with medium supplements and attachment substrates. Caution is needed when analysing data from cultured cells, as modifications of physiological properties with time could result simply from subtle changes to the culture conditions. Therefore, results relating to an experimental intervention, should be compared with time matched controls.

Cultured adult myocytes are already a useful experimental preparation, and complement other models of the in-vivo myocardium available in the field of cardiac research. The recent use of cultured myocytes for molecular biological experiments is a prime example of their value. We anticipate that cultured myocytes will become more important as molecular techniques for modifying gene expression continue to develop and improve.

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References

[1] Burrows MT. Rhythmical activity of isolated heart muscle cells in vitro. Science 1912;3:90–92.
[2] Cavanough MW. Pulsation, migration and division in dissociated chick heart cells in vitro. J Exp Zool 1955;128:573–589.
[3] Harary I, Farley B. In vitro studies of isolated beating heart cells. Science 1960;131:1674–1675.
[4] Powell T, Twist VW. A rapid technique for the isolation and purification of adult cardiac muscle cells having respiratory control and a tolerance to calcium. Biochem Biophys Res Commun 1976;72:327–333.
[5] Jacobson SL. Culture of spontaneously contracting myocardial cells from adult rats. Cell Struct Funct 1977;2:1–9.
[6] Simpson P, McGrath A, Savion S. Myocyte hypertrophy in neonatal rat heart cultures and its regulaton by serum and by catcholamines. Circ Res 1982;51:787–801.
[7] Kasten FH. Rat myocardial cells in vitro: mitosis and differentiated properties. In Vitro 1972;8:128–150.
[8] Fange R, Persson H, Thesleff S. Electrophysiologic and pharmacological observations on trypsin-disintegrated embryonic chick hearts cultured in vitro. Acta Physiol Scand 1959;38:173–183.
[9] Pollinger IS. Growth and DNA synthesis in embryonic chick heart cells, in vivo and in vitro. Exp Cell Res 1973;76:253–262.
[10] Nag AC, Crandell TF, Cheng M. Competence of embryonic mammalian heart cells in culture: DNA synthesis, mitosis, and differentiation. Cytobios 1981;30:189–208.
[11] Jacobson SL, Piper HM. Cell cultures of adult cardiomyocytes as models of the myocardium. J Mol Cell Cardiol 1986;18:661–678.
[12] Piper HM, Volz A, Schwartz P. Adult ventricular rat heart muscle cells. In: Piper HM, editors. Cell culture techniques in heart and vessel research. Berlin: Springer–Verlag, 1990:36–60.
[13] Berger HJ, Prasad SK, Davidoff AJ, et al. Continual electric field stimulation preserves contractile function of adult ventricular myocytes in primary culture. Am J Physiol Heart Circ Physiol 1994;266:H341–H349.
[14] Schackow TE, Decker RS, Ten Eick RE. Electrophysiology of adult cat cardiac ventricular myocytes: changes during primary culture. Am J Physiol Cell Physiol 1995;268:C1002–C1017.
[15] Drazner MH, Peppel KC, Dyer S, Grant AO, Koch WJ, Lefkowitz RJ. Potentiation of beta-adrenergic signaling by adenoviral-mediated gene transfer in adult rabbit ventricular myocytes. J Clin Invest 1997;99:288–296.
[16] Feng JL, Wible B, Li GR, Wang ZG, Nattel S. Antisense oligodeoxynucleotides directed against Kv1.5 mRNA specifically inhibit ultrarapid delayed rectifier K^+ current in cultured adult human atrial myocytes. Circ Res 1997;80:572–579.
[17] Borg TK, Terracio L. Attachment substrates for heart muscle cells. In: Piper HM, editors. Cell culture tecniques for heart and vessel research. Berlin: Springer–Verlag, 1990:99–107.
[18] Decker ML, BehnkeBarclay M, Cook MG, Lesch M, Decker RS. Morphometric evaluation of the contractile apparatus in primary cultures of rabbit cardiac myocytes. Circ Res 1991;69:86–94.
[19] Kirshenbaum LA, MacLellan WR, Mazur W, French BA, Schneider MD. Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus. J Clin Invest 1993;92:381–387.
[20] Shubeita HE, Thorburn J, Chien KR. Microinjection of antibodies and expression vectors into living myocardial cells: development of a novel approach to identify candidate genes that regulate cardiac growth and hypertrophy. Circulation 1992;85:2236–2246.
[21] Decker ML, BehnkeBarclay M, Cook MG, La Pres JJ, Clark WA, Decker RS. Cell shape and organization of the contractile apparatus in cultured adult cardiac myocytes. J Mol Cell Cardiol 1991;23:817–832.
[22] Haddad J, Decker ML, Hsieh LC, Lesch M, Samarel AM, Decker RS. Attachment and maintenance of adult rabbit cardiac myocytes in primary cell culture. Am J Physiol Cell Physiol 1988;255:C19–C27.
[23] Decker ML, Simpson DG, Behnke M, Cook MG, Decker RS. Morphological analysis of contracting and quiescent adult rabbit cardiac myocytes in long-term culture. Anat Rec 1990;227:285–299.
[24] Ellingsen O, Davidoff AJ, Prasad SK, et al. Adult rat ventricular myocytes cultured in defined medium: phenotype and electromechanical function. Am J Physiol Heart Circ Physiol 1993;265:H747–H754.
[25] Nag AC, Cheng M. Biochemical evidence for cellular dedifferentiation in adult rat cardiac muscle cells in culture: expression of myosin isozymes. Biochem Biophys Res Commun 1986;137:855–862.
[26] Benardeau A, Hatem SN, Ruckermartin C, et al. Primary culture of human atrial myocytes is associated with the appearance of structural and functional characteristics of immature myocardium. J Mol Cell Cardiol 1997;29:1307–1320.
[27] Bugaisky LB, Zak R. Differentiation of adult rat cardiac myocytes in cell culture. Circ Res 1989;64:493–500.
[28] Claycomb WC, Burns AH, Shepherd RE. Culture of the terminally differentiated ventricular cardiac muscle cell. Characterization of exogenous substrate oxidation and the adenylate cyclase system. FEBS Lett 1984;169:261–266.
[29] Claycomb WC, Palazzo MC. Culture of the terminally differentiated adult cardiac muscle cell: a light and scanning electron microscope study. Dev Biol 1980;80:466–482.
[30] Ikeda U, Briggs GM, Allen PD, Sen L, Medford RM, Smith TW. Properties of adult rat ventricular cells in long-term culture. Cardioscience 1990;1:225–233.
[31] Claycomb WC, Lanson Jr. N. Isolation and culture of the terminally differentiated adult mammalian ventricular cardiac muscle cell. In Vitro 1984;20:647–651.
[32] Piper HM, Probst I, Schwartz P, Hutter JF, Spieckermann PG. Culturing of calcium stable adult cardiac myocytes. J Mol Cell Cardiol 1982;14:397–412.
[33] Mitcheson JS, Hancox J, Levi AJ. Action potentials, ion channel currents and transverse tubule density in adult rabbit ventricular myocytes maintained for 6 days in cell culture. Pflug Arch 1996;431:814–827.
[34] Jacobson SL, Banfalvi M, Schwarzfeld TA. Long term primary cultures of adult human and rat cardiomyocytes. Basic Res Cardiol 1985;80(suppl 1):79–82.
[35] Allen DG, Eisner DA, Orchard CH. Characterisation of oscillations of intracellular calcium concentration in ferret ventricular muscle. J Physiol 1984;352:113–128.
[36] Wier WG, Cannell MB, Berlin JR, Marban E, Lederer WJ. Cellular and subcellular heterogeneity of $[Ca^{2+}]i$ in single heart cells revealed by Fura-2. Science 1987;235:325–328.
[37] Volz A, Piper HM, Siegmund B, Schwartz P. Longevity of adult ventricular rat heart muscle cells in serum-free primary culture. J Mol Cell Cardiol 1991;23:161–173.
[38] Piper HM, Jacobson SL, Schwartz P. Determinants of cardiomyocyte development in long-term primary culture. J Mol Cell Cardiol 1988;20:825–835.
[39] Dow JW, Harding NGL, Powell T. Isolated cardiac myocytes II Functional aspects of mature cells. Cardiovasc Res 1981;15:549–579.
[40] Stemmer P, Akera T, Brody TM, Rardon DP, Watanabe AM. Isolation and enrichment of Ca^{2+}-tolerant myocytes for biochemical experiments from guinea-pig heart. Life Sci 1989;44:1231–1237.

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[41] Tytgat J. How to isolate cardiac myocytes. Cardiovasc Res 1994;28:280–283.
[42] Cooper G, Mercer WE, Hoober JK, et al. Load regulation of the properties of adult feline cardiocytes: the role of substrate adhesion. Circ Res 1986;58:692–705.
[43] Simpson DG, Terracio L, Terracio M, Price RL, Turner DC, Borg TK. Modulation of cardiac myocyte phenotype in vitro by the composition and orientation of the extracellular matrix. J Cell Physiol 1994;161:89–105.
[44] Hilenski LL, Terracio L, Sawyer R, Borg TK. Effects of extracellular matrix on cytoskeletal and myofibrillar organization in vitro. Scanning Microsc 1989;3:535–548.
[45] Lundgren E, Gullberg D, Rubin K, Borg TK, Terracio MJ, Terracio L. In vitro studies on adult cardiac myocytes: attachment and biosynthesis of collagen type IV and laminin. J Cell Physiol 1988;136:43–53.
[46] Borg TK, Rubin K, Lundgren E, Borg K, Obrink B. Recognition of extracellular matrix components by neonatal and adult cardiac myocytes. Dev Biol 1984;104:86–96.
[47] Mitcheson JS, Hancox J, Levi AJ. Isolating and culturing adult heart cells. In: Doyle A, Griffiths JB, editors. Cell and tissue culture: laboratory procedures. Chichester: Wiley, 1996:1.1–1.13.
[48] Jacobson SL, Kennedy CB, Mealing GAR. Evidence for functional sodium and calcium ion channels in the membrane of cultured cardiomyocytes of the adult rat. Can J Physiol Pharmacol 1983;61:1312–1316.
[49] Lipp P, Huser J, Pott L, Niggli E. Spatially non-uniform Ca signals induced by the reduction of transverse tubules in citrate loaded guinea-pig ventricular mycoyes in culture. J Physiol 1996;497(3):589–597.
[50] Kato S, Ivester CT, Cooper G, Zile MR, McDermott PJ. Growth effects of electrically stimulated contraction of adult feline cardiocytes in primary culture. Am J Physiol 1995;268:H2495–H2504.
[51] Rybicka K. Glycosomes (protein–glycogen complex) in the canine heart. Virchows Arch (Cell Pathol) 1979;30:335–347.
[52] Horackova M. The effect of stretch on the adaptation of adult guinea-pig ventricular myocytes in culture: cytoarchitecture visualized by confocal microscopy. Biophys J 1997;72:WP160.
[53] Sommer JR, Johnson EA. A comparative ultrastructure of cardiac membrane specializations. A review. Am J Cardiol 1970;25:184–194.
[54] Mitcheson JS, Hancox J, Levi AJ. Cultured adult rabbit myocytes: effect of adding supplements to the medium, and response to isoprenaline. J Cardiovasc Electrophysiol 1997;8:1020–1030.
[55] Rohrer DK, Hartong R, Dillmann WH. Influence of thyroid hormone and retinoic acid on slow sarcoplasmic reticulum Ca ATPase and myosin heavy chain a-gene expression in cardiac myocytes. Delineation of *cis*-active DNA elements that confer responsiveness to thyroid hormone but not to retinoic acid. J Biol Chem 1991;266:8638–8646.
[56] Barry DM, Nerbonne JM. Myocardial potassium channels: electrophysiological and molecular diversity. Annu Rev Physiol 1996;58:363–394.
[57] Deal KK, England SK, Tamkun MM. Molecular physiology of cardiac potassium channels. Physiol Rev 1996;76(1):49–67.
[58] Janvier NC, Boyett MR. The role of Na–Ca exchange current in the cardiac action potential. Cardiovasc Res 1996;32:69–84.
[59] Isenberg G, Klockner U. Isolated bovine ventricular myocytes: characterisation of the action potential. Pflug Arch 1982;395:19–29.
[60] Isenberg G, Klockner U. Calcium currents of isolated bovine ventricular myocytes are fast and of large amplitude. Pflug Arch 1982;395:30–41.
[61] Cannell MB, Berlin JR, Lederer WJ. Intracellular calcium in cardiac myocytes: calcium transients measured using fluorescence imaging. In: Mandel LJ, Eaton DC, editors. Cell calcium and the control of membrane transport. New York: The Rockefeller University Press, 1987:201–214.
[62] Wier WG. [Ca^{2+}]i transients during excitation–contraction coupling of mammalian heart. In: Fozzard HA, Haber E, Jennings RB, Katz AM, editors. The heart and cardiovascular system. New York: Raven Press, 1992:1223–1248.
[63] Ten Eick RE, Whalley DW, Rasmussen HH. Connections: heart disease, cellular electrophysiology, and ion channels. FASEB J 1992;6:2568–2580.
[64] Hatem SN, Benardeau A, Rucker-Martin C, Samuel JL, Caroboeuf E, Mercadier JJ. Differential regulation of voltage activated potassium currents in cultured human atrial myocytes. Am J Physiol 1996;271:H1609–H1619.
[65] Feng JL, Li G, Fermini B, Nattel S. Properties of sodium and potassium curents of cultured human atrial myocytes. Am J Physiol 1996;270:H1676–H1686.
[66] Cerbai E, Barbieri M, Li Q, Mugelli A. Ionic basis of action potential prolongation of hypertrophied cardiac myocytes isolated from hypertensive rats of different ages. Cardiovasc Res 1994;28:1180–1187.
[67] Tomita F, Bassett AL, Myerburg RJ, Kimura S. Diminished transient outward currents in rat hypertrophied ventricular myocytes. Circ Res 1994;75:296–303.
[68] Fiset C, Clark RB, Shimoni Y, Giles WR. *Shal*-type channels contribute to the Ca^{2+}-independent transient outward K^+ current in rat ventricle. J Physiol 1997;500:51–64.
[69] England SK, Uebele VN, Shear H, Kodali J, Bennett PB, Tamkun MM. Characterization of a voltage-gated K^+ channel beta subunit expressed in human heart. Proc Natl Acad Sci USA 1995;92:6309–6313.
[70] Brown AM. Structure and function of cardiac Na, Ca and K channels. In: Roberts R, editor. Molecular basis of cardiology. Oxford: Blackwell, 1993:239–267.
[71] Davidoff AJ, Maki TM, Ellingsen O, Marsh JD. Expression of calcium channels in adult cardiac myocytes is regulated by calcium. J Mol Cell Cardiol 1997;29:1791–1803.
[72] Kaye D, Pimental D, Prasad S, et al. Role of transiently altered sarcolemmal membrane permeability and basic fibroblast growth factor release in the hypertrophic response of adult rat ventricular myocytes to increased mechanical activity in vitro. J Clin Invest 1996;97(2):281–291.
[73] Mann DL, Kent RL, Cooper G. Load regulation of the properties of adult feline cardiocytes: growth induction by cellular deformation. Circ Res 1989;64:1079–1090.
[74] Ivester CT, Kent RL, Tagawa H, et al. Electrically stimulated contraction accelerates protein synthesis rates in adult feline cardiocytes. Am J Physiol Heart Circ Physiol 1993;265:H666–H674.
[75] Clark WA, Rudnick SJ, LaPres JJ, Lesch M, Decker RS. Hypertrophy of isolated adult feline heart cells following beta-adrenergic-induced beating. Am J Physiol Cell Physiol 1991;261:C530–C542.
[76] Postlethwait JH, Talbot WS. Zebrafish genomics: from mutants to genes. Trends Genet 1997;13(5):183–190.
[77] Roush W. A zebrafish genome project?. Science 1997;275:923.
[78] Eisen JS. Zebrafish make a big splash. Cell 1996;876:969–977.
[79] Stainier DYR, Fouquet B, Chen J, et al. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. Development 1996;123:285–292.
[80] Gelb BD. Molecular genetics of congenital heart disease. Curr Opin Cardiol 1997;12:321–328.
[81] Baker K, Warren KS, Yellen G, Fishman MC. Defective "pacemaker" current (I-h) in a zebrafish mutant with a slow heart rate. Proc Natl Acad Sci USA 1997;94:4554–4559.
[82] Robbins J. Altering cardiac function via transgenesis a nuts and bolts approach. Trends Cardiovasc Med 1997;7:185–191.
[83] Franz W, Mueller OJ, Hartong R, Frey N, Katus HA. Transgenic animal models: new avenues in cardiovascular physiology. J Mol Med 1997;75:115–129.
[84] Ben-Yehuda O, Rockman HA. Regulation of myocardial contractili-

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ty: insights from transgenic mice. Trends Cardiovasc Med 1996;6:95–99.
[85] Rockman HA. Uncoupling of G-Protein coupled receptors in vivo: insights from transgenic mice. Adv Exp Med Biol 1997;430:67–72.
[86] Hunter JJ, Zho H, Lee KJ, Kubalak S, Chien KR. Targeting gene expression to specific cardiovascular cell types in transgenic mice. Hypertension 1993;22:608–617.
[87] Nuss HB, Marban E. Electrophysiological properties of neonatal mouse cardiac myocytes in primary culture. J Physiol 1994;479:265–279.
[88] Gomez JP, Potreau D, Branka J, Raymond G. Developmental changes in Ca^{2+} currents from newborn rat cardiomyocytes in primary culture. Pflug Arch Eur J Physiol 1994;428:241–249.
[89] Gomez JP, Potreau D, Raymond G. Intracellular calcium transients from newborn rat cardiomyocytes in primary culture. Cell Calcium 1994;15:265–275.
[90] Price RL, Nakagawa M, Terracio L, Borg TK. Ultrastructural localization of laminin on in vivo embryonic, neonatal, and adult rat cardiac myocytes and in early rat embryos raised in whole-embryo culture. J Histochem Cytochem 1992;40:1373–1381.
[91] Post JA, Kuwata JH, Langer GA. A discrete Na^{+}/Ca^{2+} exchange dependent, Ca^{2+} compartment in cultured neonatal rat heart cells. Characteristics, localization and possible physiological function. Cell Calcium 1993;14:61–71.
[92] Haworth RA, Griffin P, Saleh B, Goknur AB, Berkoff HA. Contractile function of isolated young and adult rat heart cells. Am J Physiol 1987;253:H1484–H1491.
[93] Wibo M, Bravo G, Godfraind T. Postnatal maturation of excitation–contraction coupling in rat ventricle in relation to the subcellular localisation and surface density of 1,4, dihydropyridine and ryanodine receptors. Circ Res 1991;68:662–673.
[94] Boerth SR, Zimmer DB, Artman M. Steady-state mRNA levels of the sarcolemmal Na^{+}–Ca^{2+} exchanger peak near birth in developing rabbit and rat hearts. Circ Res 1994;74:354–359.
[95] Cohen NM, Lederer WJ. Changes in the calcium current of rat heart ventricular myocytes during development. J Physiol 1988;406:115–146.
[96] Wobus AM, Wallukat G, Hescheler J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation 1991;48:173–182.
[97] Forbes MS, Hawkey LA, Sperelakis N. The transverse-axial tubular system (TATS) of mouse myocardium: its morphology in the developing and adult animal. Am J Anat 1984;170:143–162.
[98] Christensen G, Wang Y, Chien KR. Physiological assessment of complex cardiac phenotypes in genetically engineered mice. Am J Physiol 1997;272:H2513–H2524.
[99] Harrer JM, Haghighi K, Hae Won K, Ferguson DG, Kranias EG. Coordinate regulation of SR Ca^{2+}–ATPase and phospholamban expression in developing murine heart. Am J Physiol 1997;272:H57–H66.
[100] Berul CI, Aronovitz MJ, Wang PJ, Mendelsohn ME. In vivo cardiac electrophysiology studies in the mouse. Circulation 1996;94:2641–2648.
[101] Kaplan ML, Cheslow Y, Vikstrom K, et al. Cardiac adaptations to chronic exercise in mice. Am J Physiol 1994;267:H1167–H1173.
[102] Rockman HA, Ono S, Ross RS, et al. Molecular and physiological alterations in murine ventricular dysfunction. Proc Natl Acad Sci USA 1994;91:2694–2698.
[103] Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. Nature 1994;372:333–335.
[104] Delcarpio JB, Lanson Jr. NA, Field LJ, Claycomb WC. Morphological characterization of cardiomyocytes isolated from a transplantable cardiac tumor derived from transgenic mouse atria (AT-1 cells). Circ Res 1991;69:1591–1600.
[105] Steinhelper ME, Lanson Jr. NA, Dresdner KP, et al. Proliferation in vivo and in culture of differentiated adult atrial cardiomyocytes from transgenic mice. Am J Physiol Heart Circ Physiol 1990;259:H1826–H1834.
[106] Lanson Jr. NA, Glembotski CC, Steinhelper ME, Field LJ, Claycomb WC. Gene expression and atrial natriuretic factor processing and secretion in cultured AT-1 cardiac myocytes. Circulation 1992;85:1835–1841.
[107] Habara-Ohkubo A. Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells. Cell Struct Funct 1996;21:101–110.
[108] Eisenberg CA, Bader D. QCE-6: a clonal cell line with cardiac myogenic and endothelial cell potentials. Dev Biol 1995;167:469–481.
[109] Morton ME, Brumwell C, Gartside CL, Hauschka SD, Nathanson NM. Characterization of muscarinic acetylcholine receptors expressed by an atrial cell line derived from a transgenic mouse tumor. Circ Res 1994;74:752–756.
[110] Sculptoreanu A, Morton M, Gartside CL, Hauschka SD, Catterall WA, Scheuer T. Tetrodotoxin-insensitive sodium channels in a cardiac cell line from a transgenic mouse. Am J Physiol 1992;262:C724–C730.
[111] Sipido KR, Marban Jr E. L-type calcium channels, potassium channels, and novel nonspecific cation channels in a clonal muscle cell line derived from embryonic rat ventricle. Circ Res 1991;69:1487–1499.
[112] Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res 1991;69:1476–1486.
[113] Wang Y, Neckelmann N, Mayne A, et al. Establishment of a human fetal cardiac myocyte cell line. In Vitro Cell Dev Biol 1991;27:63–74.
[114] Borchgrevink PC, Ryan MP. Effects of magnesium, ouabain and bumetanide on ^{86}rubidium uptake in a human atrial cell line. Br J Pharmacol 1988;95:614–618.
[115] Gollasch M, Kleuss C, Hescheler J, Wittig B, Schultz G. G(i2) and protein kinase C are required for thyrotrophin-releasing hormone-induced stimulation of voltage-dependent Ca channels in rat pituitary GH3 cells. Proc Natl Acad Sci USA 1993;90:6265–6269.
[116] Kariya K, Karns L, Simpson PC. Expression of a constituitively activated mutant of the beta-isozyme of protein kinase-C in cardiac myocytes stimulates the promoter of the beta-myosin heavy chain isogene. J Biol Chem 1991;266:10023–100236.
[117] Mestril R, Chi S, Sayen MR, O'Reilly K, Dillman WH. Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. J Clin Invest 1994;93:759–767.
[118] Paradis P, MacLellan WR, Belaguli NS, Schwartz RJ, Schneider MD. Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. J Biol Chem 1996;271:10827–10833.
[119] Kirshenbaum LA, Abdellatif M, Chakraborty S, Schneider MD. Human E2F-1 reactivates cell cycle progression in ventricular myocytes and represses cardiac gene transcription. Dev Biol 1996;179:402–411.
[120] Ellison KE, Bishopric NH, Webster KA, et al. Fusigenic liposome-mediated DNA transfer into cardiac myocytes. J Mol Cell Cardiol 1996;28:1385–1399.
[121] Schackow TE, Sheets MF, Decker RS, Teneick RE. Alteration of the sodium current in cat cardiac ventricular myocytes during primary culture. Am J Physiol 1995;37:C993–C1001.

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