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Structural and functional plasticity in long-term cultures of adult ventricular myocytes

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ABSTRACT

Cultured heart cells have long been valuable for characterizing biological mechanism and disease pathogenesis. However, these preparations have limitations, relating to immaturity in key properties like excitation–contraction coupling and β-adrenergic stimulation. Progressive attenuation of the latter is intimately related to pathogenesis and therapy in heart failure. Highly valuable would be a long-term culture system that emulates the structural and functional changes that accompany disease and development, while concurrently permitting ready access to underlying molecular events. Accordingly, we here produce functional monolayers of adult guinea-pig ventricular myocytes (aGPVMs) that can be maintained in long-term culture for several weeks. At baseline, these monolayers exhibit considerable myofibrillar organization and a significant contribution of sarcoplasmic reticular (SR) Ca^{2+} release to global Ca^{2+} transients. In terms of electrical signaling, these monolayers support propagated electrical activity and manifest monophasic restitution of action-potential duration and conduction velocity. Intriguingly, β-adrenergic stimulation increases chronotropy but not inotropy, indicating selective maintenance of β-adrenergic signaling. It is interesting that this overall phenotypic profile is not fixed, but can be readily enhanced by chronic electrical stimulation of cultures. This simple environmental cue significantly enhances myofibrillar organization as well as β-adrenergic sensitivity. In particular, the chronotropic response increases, and an inotropic effect now emerges, mimicking a reversal of the progression seen in heart failure. Thus, these aGPVM monolayer cultures offer a valuable platform for clarifying long elusive features of β-adrenergic signaling and its plasticity.

1. Introduction

Cultured heart cell preparations have proven to be an invaluable platform for the in-depth examination of biological mechanism, pathogenesis, and proof-of-principle therapy [1–6]. These models allow convenient observation and manipulation of numerous physiological mechanisms. Among the most important properties rendered accessible for scrutiny are sarcomeric myofibrillar architecture and excitation–contraction (E–C) coupling, action-potential propagation and morphology, and β-adrenergic signaling. These dimensions bear respectively on Ca^{2+} cycling and dysfunction, arrhythmogenesis, and the evolution of heart failure [7–9]. The latter malady is intimately mirrored by gradual desensitization of such signaling, while pharmacological blockade of the β-adrenergic cascade helps counter disease progression [10].

Immature myocytes have been the most popular starting source for cultures, because these cells are plentiful and typically yield preparations with lifespans of weeks or longer, permitting convenient genetic manipulability by viral transduction. In particular, commonly used cells include ventricular myocytes from neonatal/embryonic rat [11–15] and mouse [16,17], and more recently from human stem-cell-derived cardiomyocytes [18–20]. The comparative ease of engineering transgenic mice adds to the popularity of the murine source [16], and immature cells from all of these origins can readily form planar monolayers or even three-dimensional configurations, allowing examination of higher-order network properties. Thus, phenomena like electrical wave propagation relating to arrhythmogenesis may be deconstructed with considerable resolution.

At the same time, cultures from these cell sources present significant limitations. Firstly, myofibrillar organization in these cultures can be incomplete, and related E–C coupling properties rather immature [19,21,22]. Secondly, cardiac action potential (AP) morphologies in rats and mice differ considerably from humans, in that the phase-2 plateau is largely lacking in these rodents [23], hampering their use as models for short- and long-QT-related human phenomena such as the genesis of early after depolarizations. This difference may reflect a predominance of rapidly activating K^+ currents in the cardiomyocytes of these small rodents [24]. Thirdly, β-adrenergic signaling in these preparations is incomplete compared with adult cardiomyocytes,

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often featuring only chronotropic but not inotropic actions [19,25,26], in the absence of elaborate tissue engineering cues [20,27]. In a sense, these cultures may reflect the immaturity of the source cells themselves. Finally, although preparations derived from human stem cells feature somewhat longer APs reminiscent of adult humans; they nonetheless lack mature β-adrenergic signaling and E–C coupling [19,26,28]. They are also comprised of a pleiotropic mixture of immature precursors to various cardiac cell types [29,30].

Alternatives of considerable promise are cultures derived from adult cardiac myocytes, specifically from species featuring prominent phase-2 morphology, as well as robust chronotropic and inotropic responses to β-adrenergic stimulation [31] (e.g., guinea pigs, rabbits, and dogs). Acutely isolated myocytes from these sources are a mainstay of biophysical inquiry, but cultures derived from these cells have generally been associated with limited longevity, lasting less than a week [32,33]. Yet, there have been reports of long-term cultures of adult cardiomyocytes [34], particularly from guinea pig [35,36], rat [1,37], and rabbit [38–40]. Indeed, guinea-pig preparations can even form confluent monolayers [35,41]. Because of the adult origin of these cultured myocytes, there is hope that these cultures may recapitulate a more mature phenotype. To be specific, adult cells in culture initially undergo partial dematuration and subsequent rematuration [1,5,33,41]. After rematuration, however, some cultures do exhibit clearly visualized striations, fueling expectations that a mature profile has been partially restored [1,41]. Despite such potential, the extent to which these adult-derived, long-term cultures maintain mature E–C coupling and organization, adult-like AP morphology, and robust β-adrenergic responsiveness has received comparatively little study. Moreover, the functional tissue properties of such adult-cardiomyocyte-derived preparations, when reassembled as monolayers, remain unknown. Finally, largely unexplored is the potential of these cultures to adopt a more mature phenotype, upon exposure to appropriate environmental cues.

Here, we therefore scrutinize the morphological and functional characteristics of long-term cultures derived from adult guinea-pig ventricular myocytes (aGPVMs). A guinea-pig source is chosen because of the robust AP plateau phases and β-adrenergic signaling in these animals [31]. We form these cultures into monolayers, permitting exploration of myofibrillar organization and E–C coupling, AP propagation and morphology, and β-adrenergic responsiveness. We also demonstrate straightforward genetic manipulability by viral-mediated expression of auxiliary beta subunits of voltage-gated calcium channels, which markedly alters AP morphology. Most notably, we provide evidence that long-term aGPVM cultures possess considerable potential for additional maturation by an appropriate external prompt. Simple application of repetitive electrical stimulation over one week in culture [42,43] improves spatial organization of contractile proteins. More intriguing is the manner of enhancement of β-adrenergic stimulation. Whereas baseline cultures (without long-term electrical stimulation) demonstrate chronotropic but not inotropic responses, cultures exposed to prolonged electrical stimuli exhibit not only stronger chronotropic enhancement, but also clear reemergence of inotropic response. This preparation therefore offers a powerful and convenient system for exploring the molecular underpinnings of specific limbs of the β-adrenergic response, whose selective loss and gain parallels heart failure progression and treatment [10,44]. Applying other environmental cues drawn from a burgeoning tissue-engineering toolkit will likely yield further maturation of this promising preparation. A portion of this study was previously presented [45].

2. Methods

2.1. Isolation and long-term culture of aGPVMs

The perfusion and isolation of adult guinea-pig ventricular myocytes were performed according to published protocols [46]. In brief, single ventricular myocytes were isolated from whole hearts of adult male guinea pigs (Hartley strain, 3–4 wk old, weight 250–350 g). After excision, the heart was placed in cold (4 °C) Tyrode's solution (Ca^{2+} free) to remove blood and was mounted onto a Langendorff apparatus, and perfused retrogradely with the following three oxygenated solutions in sequential order: 1) modified Tyrode's buffer pH 7.5 containing (in M) NaCl (0.14), $MgCl_2$ (0.001), HEPES (0.01), KCl (0.005) and glucose (0.01); 2) digestion enzyme solution in Tyrode buffer pH 7.5, containing collagenase type 2 (78.13 U/ml) (Worthington CLS-2, cat no. LS004177) and protease XIV (0.37 U/ml) (Sigma P5147); 3) high K solution (pH 7.4) containing (in M) K-glutamate (0.12), KCl (0.025), HEPES (0.01), $MgCl_2$ (0.001) and EGTA (0.001). Following perfusion the heart was excised and placed in a sterile high K-buffer in a petri dish and transferred to a laminar flow hood. The tissue was minced into smaller pieces and suspended several times to dissociate the cells. The cell suspension was filtered through a mesh and allowed to sediment for 15 min. The pellet was suspended in 10% M199 media and observed under a microscope.

For long-term culture, we used a method modified from Horackova et al. [47]. Plastic or glass cover slips were coated with laminin (20 μg/ml overnight at 4 °C) and placed in 12 well tissue culture dishes (Becton Dickinson, NJ, USA). The dissociated adult guinea-pig cardiomyocytes were suspended in M199 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-Glutamine (Gibco), and MEM non-essential amino acids (Gibco). We found that the key to make a monolayer was to use a high density (10^5 cells/ml) of rod-shaped cells during plating (60,000 cells/cm^2 plating density). Cell–cell contact affects the hypertrophic response of the cells and the differentiated phenotype, and aids in cell survival [2]. Accordingly, guinea pig cardiomyocytes were plated at a cell density of 2×10^5 cells/ml on 20 mm coverslips placed in tissue culture dishes. The cells were allowed to settle and attach at 37 °C overnight in an atmosphere of 5% CO_2–95% air. 24 h post incubation, fresh M199 plus 10% FBS was added, and cells were cultured for 48 h. The culture medium was then changed to M199 plus 5% FBS for the next 72 h. Following that, the medium was changed every other day to M199 plus 1% FBS, for the next three weeks. The inclusion of FBS was necessary for maintenance of monolayer cultures beyond ~1.5 weeks [1]. Cultures were periodically monitored for viability and confluency for a period of up to 5 weeks.

2.2. Electrical stimulation of aGPVM monolayers

Electrical stimulation was initiated 10–14 days after plating, once confluency was attained in the monolayer. Electrical stimulation was then applied for up to 21 days. Trains of electrical stimuli (4 ms duration, 1.5 Hz, 10 V/cm) were delivered using a C-Pace EP Culture Pacer (IonOptix, LLC, MA, USA), which likely impart increased repetitive regularity in syncytial contraction during culture [42,48]. aGPVM cultures without electrical stimulation under identical conditions were included as 'nonstimulated' controls.

2.3. Lentivirus or adenovirus transduction of aGPVMs

Adenoviral vectors for the β_{2a} subunit were generated as previously described [49]. The lentiviral vectors for the β_{2a} subunit were generated by using third-generation constructs as previously reported [50]. The cDNA for rat β_{2a} (GenBank accession no. M80545) was subcloned into the lentiviral plasmid, pRRLsin18.cPPT.CMV.eGFP.Wpre (designated as LV-eGFP). The resulting plasmid, pPPT.CMV.β_{2a}GFP was designated as LV-β_{2a}GFP. LV was produced by calcium-phosphate transfection of HEK293 cells, followed by column-based purification and titering via previously described protocols [50].

aGPVMs at 19 days post-plating were infected with Adv-β_{2a}GFP, and 24 h post-infection the medium was aspirated and replaced with fresh M199 medium containing 1% serum. 24 h later, the expression of Adv-β_{2a}GFP was ~90% in aGPVMs. Alternatively, aGPVMs at 10 days