Pro-arrhythmogenic potential of immature cardiomyocytes is triggered by low coupling and cluster size

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Abstract

Objective: Cell transplantation strategies to regenerate compromised myocardium take advantage of in vitro generated cardiomyocytes. Common in those immature myocytes is spontaneous impulse formation and a restricted ability to establish proper electrical interaction. Spontaneous impulse formation and impaired cell-to-cell coupling have been shown to be arrhythmogenic. To investigate whether these features harbor a pro-arrhythmic potential for cell transplantation, a co-culture of spontaneously active neonatal rat cardiomyocytes (NRC) and quiescent adult dog cardiomyocytes (ADC) was used.

Methods: ADCs and NRCs were isolated and cultured on laminin-coated substrates. Connexin43, N-cadherin and α-actinin expression was evaluated with immunohistochemistry. Intercellular coupling was measured in cell pairs using the dual voltage clamp technique and fluorescent dye injection.

Results: One day after isolation, NRCs were beating spontaneously, while ADCs remained quiescent in monoculture. ADC resting membrane potential was −80.3 ± 0.2 mV (mean ± SEM, N = 24) and did not change significantly over time. NRCs had a maximal diastolic potential of −65.0 ± 2.8 mV (N = 4). After one day of co-culture, pseudopodia-like extensions developed at the former intercalated discs of ADCs, contacting the NRCs. Only ADCs that contacted three or more NRCs started to beat in synchrony. Expression of connexin43 and N-cadherin indicated presence of electrical and mechanical junctions at the interface between the two cell-types. Transfer of Lucifer Yellow demonstrated junctional permeability between ADCs and NRCs. Junctional conductance between ADC–ADC (31.9 ± 5.1 nS, N = 10) and NRC–NRC (35.0 ± 9.6 nS, N = 6) pairs was significantly higher compared to ADC–NRC pairs (9.7 ± 2.9 nS, N = 8). Gap-junctional blockade with halothane reversibly abolished NRC-triggered beating of ADCs. Computer simulations demonstrated that within a delicate ‘window’ of gap junctional conductance small clusters of spontaneously active cells are able to induce triggered activity in quiescent mature myocytes but also in a two-dimensional sheet of ventricular cells.

Conclusion: Spontaneously active immature cardiomyocytes are able to trigger mature cardiomyocytes depending on their level of electrical coupling and the amount of coupled immature myocytes.

Keywords: Cell culture; Arrhythmia (mechanisms); Gap junctions; Cardiomyocyte; Computer modelling

1. Introduction

Spontaneous depolarization of autorhythmic myocytes within the sinoatrial (SA) node triggers each subsequent heartbeat. These nodal myocytes have no primary contractile
function but are crucial in impulse formation. They lack a stable resting membrane potential and exhibit a spontaneous diastolic depolarization. This electrical instability is primarily due to the low amount of channels conducting the (stabilizing) inward rectifier current ($I_{K1}$). In the SA node, the level of intercellular electrical coupling, as mediated by gap junction channels, is low between myocytes within the compact node but this gradually increases towards the border zone with the atrial working myocardium [1]. This configuration allows the impulse of the spontaneously beating cells to exit the node and prevents clamping of the membrane potential of spontaneously active cells to the membrane potential of the atrial cells.

In the working myocardium, a proper and timely activation of all composing cardiomyocytes is effectuated by high levels of rather homogeneously expressed ion channel and gap junction channel constituents. Cardiomyocytes of the working myocardium are non-autorhythmic due to a high expression level of channels conducting $I_{K1}$. Automaticity, however, can be introduced by severe down-regulation of Kir2.1 protein; the major constituent of the $I_{K1}$ channel [2]. Electrical coupling is facilitated by gap junction channels which predominantly are constituted from hexagonally arranged connexin43 (Cx43) protein subunits [3]. Pathophysiological conditions, however, initiate irregularities in expression and distribution of gap junctions throughout the heart (meaning that part of the myocytes are not or poorly coupled to each other). Next to uncontrolled automaticity, these changes have been recognized as an important factor predisposing the heart to arrhythmias [4,5].

In order to ameliorate cardiac performance of hearts compromised by infarction or myopathy, transplantation strategies using in vitro generated donor (non)-cardiomyocytes have gained major interest. Several sources of stem cells have successfully been differentiated into stem cell derived cardiomyocytes (SDCs) but transplantation has also been performed using mesenchymal stem cells, skeletal myoblasts or even neonatal cardiomyocytes. In vitro generated cardiomyocytes share important features with nodal cardiomyocytes. Both neonatal cardiomyocytes and SDCs are spontaneously beating immature myocytes. This automaticity can be beneficial if a biological pacemaker is requested, as demonstrated by Kehat et al., who successfully introduced an ectopic pacemaker in AV-block pigs using SDCs [6]. Potapova et al. showed that transplantation of mesenchymal stem cells overexpressing HCN2, a member of the HCN ion channel subunit family underlying the cardiac ‘pacemaker current’ $I_{p}$, were similarly potent to induce pacemaking activity in a dog model [7]. However, if there is no need for new pacemaking, automaticity of immature cells may disturb the vulnerable impulse propagation and enhance the propensity to generate arrhythmias. Besides automaticity, studies on SDCs consistently report low levels of connexin expression, both in vitro and in vivo, with some cells that do and others that do not express connexins [8,9]. In clinical trials, autologous transplantation of skeletal myoblasts which also do not express Cx43 upon differentiation into excitable myotubes, resulted in ventricular tachycardia in several patients [10]. An in vitro study showed that this could be overcome by genetically induced overexpression of Cx43 [11].

From those studies, we suggest that the potential of immature myocytes to trigger the mature working myocardium not only depends on their spontaneous activity, but also on the level of established electrical coupling in analogy with the pacemaking behaviour of autorhythmic cells in the cardiac conduction system [1]. To study those features in detail, we generated an accessible in vitro approach in which we co-cultured immature and mature cardiomyocytes. Furthermore, we performed computer simulations to investigate the basic electrophysiological characteristics of their interaction. Our data show that the ability of immature cardiomyocytes to trigger quiescent mature cardiomyocytes depends on the amount of connected cells and the level of electrical coupling.

2. Materials and methods

2.1. Cell culture

Neonatal rat ventricular cardiomyocytes (NRCs) and left ventricular myocytes from adult dogs (ADCs) were isolated as described before [12,13]. The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the institutional committee for animal experiments. Isolated ADCs, collected in Tyrode supplemented with 0.2 mmol/L CaCl$_2$, were centrifuged at 400 rpm for 3 min. All but the last 10 mL supernatant was removed and 10 mL Tyrode supplemented with 0.6 mmol/L CaCl$_2$ was added. Cells were gently resuspended and centrifuged again. These steps were repeated with Tyrode containing 0.8 mmol/L and 1.0 mmol/L CaCl$_2$, respectively. Finally, cells were resuspended in DMEM without additives and gently loaded on top of 10 mL DMEM supplemented with 4% BSA in order to pellet rod-shaped ADCs by gravity. After 5 min, supernatant was removed and cells were resuspended in culture medium: DMEM supplemented with 10% fetal calf serum, 100 IU penicillin/mL and 100 μg streptomycin/mL. After a last centrifugation, cells were collected in 4–8 mL culture medium and seeded on glass coverslips coated with laminin (20 μg/mL). For co-cultures, isolated ADCs were added (4000/cm$^2$) to cultures of NRCs which were seeded one day before ADC isolation. To generate different ratios between NRCs and ADCs, NRCs were seeded at densities of 3250, 6500 and 13000 cells/cm$^2$. Medium was replaced the next day and every second day thereafter.

2.2. Immunohistochemistry

Cells were fixed with methanol at −20°C for 2 min and rinsed three times with PBS. Immunolabelling was
performed as described before [9]. Primary antibodies used were anti-Cx43 (Zymed), anti-α-actinin (Sigma) and anti-N-cadherin (Sigma).

2.3. Reverse transcriptase-polymerase chain reaction

RNA was isolated by use of Trizol (Invitrogen, Breda, The Netherlands) and DNase-I treated RNA was reverse transcribed using oligo-dT Superscript 3 (Invitrogen). The following primer pairs and PCR conditions were used: GAPDH; forward ATGACAACTCCTCAGATTGT, reverse CATTGTCATACCGAATGAG, annealing temperature 53 °C, 30 cycles; KCNJ2; forward CCCGAGAAGGTCAACATCTTG GG, reverse TCGTACGAGATCTTGACGGG, annealing temperature 60 °C, 35 cycles; SCN5A; forward CCGAGAAGGTCAACATCTTG GG, reverse TCGTACGAGATCTTGACGGG, annealing temperature 60 °C, 35 cycles. Products were analyzed on ethidium bromide-stained 1% agarose gel. GAPDH was used as RNA input control.

2.4. Electrophysiology

A symmetrical setup with two HEKA EPC-7 patch clamp amplifiers was used to measure electrical coupling between cells. All measurements were done on cells cultured for 2 days. Macroscopic gap junctional currents were recorded using a custom data acquisition program (kindly provided by J.G. Zegers, AMC, Amsterdam) running on a G4 Apple Macintosh computer equipped with a 12-bit National Instruments PCI-MIO-16E-4 acquisition card. Current signals were low-pass filtered at 2.5 kHz and acquired at 10 kHz. Macroscopic gap junctional currents were elicited in cell pairs by applying small transjunctional voltage steps (+10 mV) from holding potential (−40 mV). By using small pulses, junctional conductances were maximal and not inactivating. Offline analysis was done using MacDaq 8.0 (kindly provided by Dr. A.C.G. van Ginneken, AMC, Amsterdam) and R 2.0.1 [14]. Gap junctional conductance (g j) was defined as g j = I j / V j, where I j and V j denote junctional current and transjunctional voltage, respectively. Action potentials were elicited with a brief square current pulse. Maximal upstroke velocities were determined by differentiating the voltage traces.

Resting membrane potential, maximum upstroke velocity and I K1 density of single isolated ADCs were measured using the whole-cell mode of the patch clamp configuration. A conventional mono-exponential fitting procedure was used to derive membrane capacitance from currents elicited by −10 and +10 mV voltage clamp steps from resting membrane potential. I K1 was elicited by applying 1-second square test pulses ranging between −80 and +90 mV from a holding potential of −40 mV. Steady state currents at the end of the pulse were normalized to membrane capacitance and plotted against test pulse potential. Resulting current densities in the −130 to −80 mV voltage range were fitted with linear regression to obtain I K1 conductance density. All electrophysiological experiments were done at 20 °C. Extracellular buffer used was a modified Tyrode’s solution, containing (in mmol/L) NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1, HEPES 15, NaHCO3 35, glucose 6, pH 7.20/NaOH. Gap junctional coupling was inhibited by halothane [15]. Halothane dissolved in extracellular buffer (final concentration 4.25 mM) was applied at the interface between ADC and NRCs with an additional micropipette. Pipette buffer contained (in mmol/L) potassium gluconate 125, KCl 10, HEPES 5, EGTA 5, MgCl2 2, CaCl2 0.6, Na2ATP 4, pH 7.20/KOH. For dye injections, microelectrodes were filled with Lucifer Yellow in 150 mM/L LiCl2, 10 mM/L HEPES. Patch pipettes were pulled on a Narishige PC-10 puller and fire-polished. When filled with pipette buffer, pipette resistance ranged between 2 and 5 MΩ. Liquid junction potential was calculated using Clampex (Axon Instruments) and used for offline correction.

2.5. Computer simulations

Computer simulations were performed to assess the conditions that allow a cluster of spontaneously beating NRCs to trigger beating of an intrinsically quiescent ADC.

We used our previously published rabbit SA nodal cell model as a tentative membrane model of a spontaneously active NRC [16]. To match the membrane capacitance of a single NRC, membrane capacitance of the model was reduced by a factor of 2 to 16 pF while retaining its membrane current densities. ‘NRC cluster size’ was varied by increasing the membrane capacitance of the SA nodal cell model in steps of 16 pF instead of adding individual 16-pF cells. The rationale for doing so is that the experimentally observed gap junctional conductance between NRCs is so high that the cells of an SA nodal cell cluster show complete waveform entrainment [17]. The phase-2 Luo–Rudy mammalian ventricular cell model [18], with a membrane capacitance of 153.4 pF, was used as membrane model for ADCs. The ‘NRC cluster’ was coupled to the ‘ADC’ at a variable ohmic coupling conductance. For numerical integration, we used an Euler-type scheme with a fixed time step of 10 μs. All simulations were run for a sufficiently long time to reach steady-state behaviour.

In addition, computer simulations were performed with a two-dimensional sheet composed of 27 × 27 = 729 cells with a spontaneously active ‘focus’ located in the centre of the sheet, as previously described in detail [19]. Each cell of the square grid was connected to its neighbours through an ohmic coupling conductance. In the present study, the ‘focus’ represents a cluster of spontaneously beating NRCs. For this simulation, similar settings were used as the above described triggering of single ADCs.

2.6. Statistics

Group comparisons were made using one-way ANOVA with the Holm–Sidak post-hoc test for multiple comparisons.
Statistical significance was assumed if $P<0.05$. All data are presented as mean±SEM.

3. Results

3.1. Characterization of ADC phenotype in culture

To validate usefulness of isolated ADCs in the in vitro coculture model system, their phenotype in culture was followed. During the first 3 days, ADCs remained rod shaped with exception of small pseudopodia-like extensions that developed after days 1–2 at the former intercalated disks (IDs) on the longitudinal cell ends. ADCs developed these structures both in mono-culture and in co-culture with NRCs. Up to 3 days, α-actinin staining revealed a sharply demarcated pattern of cross-striations while former IDs largely remained intact as shown by labelling against N-cadherin (Fig. 1A, days 1 and 3). While original IDs still were abundantly positive for ID-associated proteins, like in this case N-cadherin, (Fig. 1A, day 3, arrow 1), the pseudopodia-like extensions contained additional diffuse labelling (Fig. 1A, day 3, arrow 2). At day 7 however, myocytes largely had lost their rod-shaped phenotype and compact bundled cross-striations, while IDs appeared disorganized as demonstrated by labelling against N-cadherin (Fig. 1A, day 7). Insets in Fig. 1A represent morphology of myocytes as visualized with phase contrast microscopy.

Up to 7 days, ADCs in monoculture remained quiescent. Resting membrane potential of cultured ADCs was $-80.3±0.2$ mV ($N=24$, average of 0, 1, 3 and 7 days culture-time) which did not significantly change over time (Fig. 1B). Maximal upstroke velocity of the action potential (AP) was not significantly different during the first three days but was significantly lower at day 7 (Fig. 1C). $I_{K1}$ conductance density, the main determinant of resting membrane potential and its stability [20], tended to decline with prolonged culture although this did not reach significance (Fig. 1D). Since upstroke velocity is mainly determined by sodium channel availability, we evaluated the expression level of SCN5a, the α-subunit of the cardiac sodium channel. Semi-quantitative RT-PCR revealed comparable levels of SCN5a expression at days 0, 1 and 3 while expression was slightly diminished at day 7. The expression profile of the KCNJ2 gene encoding Kir2.1 (the pore-forming unit of the $I_{K1}$ channels) revealed comparable levels throughout the culture period (Fig. 1E).

In contrast to ADCs, one day after isolation, NRCs were beating spontaneously with a maximal diastolic potential of $-65.0±2.8$ mV ($N=4$). As shown previously [21], and illustrated in Fig. 4C below, cultured NRCs did not change morphology in time. Organization of their contractile apparatus revealed strands of cross-striations as indicated by α-actinin while intense Cx43 expression was found at intercellular contacts (see Fig. 4C, right panel, below). As expected from the difference in cell size, cell membrane capacitance of ADCs was much larger than that of NRCs (184±9.7 pF, $N=18$ versus 19.6±1.4 pF, $N=19$, respectively ($P<0.05$)). During culture time, membrane capacitance of NRCs did not change significantly. Membrane capacitance of ADCs did not change during the first 3 days but was significantly increased at day 7 (336.0±47 pF, $N=6$).

![Fig. 1. A: Immunohistochemical labelling of cultured ADCs at days 1, 3 and 7 with antibodies against α-actinin (red) and N-cadherin (green). Insets represent the morphology with phase-contrast. Scale bar=25 μm. B: Resting membrane potential of ADC does not change during prolonged culture. C: Maximal upstroke velocity of triggered action potentials of ADCs in monoculture. Upstroke velocity at day 7 is significantly reduced compared to days 0 and 1 (asterisk). D: Prolonged culture did not significantly reduce $I_{K1}$ conductance density in ADCs. $N=6$ for each parameter at the subsequent days measured. E: Semi-quantitative RT-PCR data showing RNA expression levels of SCN5a and KCNJ2 at days 0, 1, 3 and 7 of cultured ADCs. GAPDH was used as RNA input control.](image-url)
3.2. Small clusters of immature cardiomyocytes can trigger a mature cardiomyocyte

Within 2 days of co-culture, through their pseudopodia-like extensions, ADCs contacted single cells or clusters of NRCs. ADC–NRC cell pairs were quiescent with slightly different membrane potentials: ADC $-79.0 \pm 2.2$ mV, NRC $-74.3 \pm 2.8$ mV ($N=4$). In contrast, ADCs contacting clusters composed of at least 3–4 spontaneously beating NRCs contracted in synchrony. Current clamp recordings of ADCs coupled to NRC clusters showed that NRCs depolarized first, indicating that the ADC was paced by the beating cluster (Fig. 2, inset). Additionally, co-cultures composed of increasing NRC-seeding densities (giving rise to larger clusters and an increased likelihood to contact ADCs) showed increasing numbers of beating ADCs. Whereas no beating ADCs were detected in co-culture with $3250$ NRCs/cm$^2$ ($N=6$), ADCs co-cultured with $6500$ ($N=4$) and $13000$ NRCs/cm$^2$ ($N=5$) showed a significantly increased incidence of beating ADCs of $28\pm10\%$ and $56\pm8\%$, respectively (Fig. 2).

3.3. Interaction between immature and mature cardiomyocytes results from de novo gap junction formation

Synchronous beating of ADCs and NRCs in our co-cultures suggested formation of functional gap junctions at the cellular interface. To confirm this, immunohistochemical labelling of $\alpha$-actinin (marking cardiomyocytes) and Cx43, the predominantly expressed gap junction-constituent in ventricular myocytes, was performed. In addition, labelling of N-cadherin representing the mechanical interaction between cells was assessed. Some diffuse Cx43 label was found in the core of the pseudopodia-like extension (Fig. 3A, arrow 1) where $\alpha$-actinin was expressed in a typical striated pattern although the organization was not comparable to that found in the remaining rod shaped part of the ADC. More importantly, at the interface between pseudopodia-like extensions from the ADC and the membrane of the NRC, a punctuate expression pattern for Cx43, indicative for genuine gap junctions, was detected (Fig. 3A, arrow 2). Even more outspoken expression patterns of N-cadherin were found at the interface between ADC and NRC. Again, diffuse labelling for N-cadherin was present in the core of pseudopodia-like extensions (Fig. 3B, arrow 1) while strong junctional labelling was found at the interface (Fig. 3B, arrow 2).

3.4. Triggered activity of ADCs occurs with poor gap junctional conductance

To test functionality of the de novo generated ADC–NRC Cx43 gap junctions, Lucifer Yellow (a 443 dalton large dye...
which transfers through Cx43 channels) was injected into ADCs. Dye spreading was observed to adjoining NRCs (N=4), confirming functional coupling of ADCs and NRCs (Fig. 4A). In order to quantify electrical conductance of gap junctions formed between NRCs and ADCs, the dual whole cell voltage clamp technique was used [22,23]. Gap junctional conductance (gj) between NRC–NRC, ADC–ADC and ADC–NRC cell pairs was compared. Average gj between NRC–NRC and ADC–ADC pairs was 35.0±9.6 nS (N=6) and 31.9±5.1 nS (N=10), respectively, which was not significantly different. Average conductance in heterotypic pairs was 9.7±2.9 nS (N=8), which was significantly lower than in both homotypic combinations (Fig. 4B). Immunohistochemical evaluation of Cx43 expression suggested that Cx43 labelling at ADC–NRC junctions (Fig. 4C, midpanel, arrow) was less pronounced as compared to that in ADC–ADC or NRC–NRC junctions (Fig. 4C, left and right panel respectively).

As mentioned, ADCs contacting sufficiently large clusters of NRCs beat in synchrony. To confirm a role for gap junctional communication in such pacing, and to exclude coupling by cytoplasmic connections, beating ADCs were uncoupled from coupled NRCs by application of halothane (N=4). Current clamp experiments showed that when uncoupled, ADCs stopped beating in synchrony with NRCs. Meanwhile their membrane potential became more negative approaching values comparable to those recorded in single cell ADCs. At the same time, the membrane potential of uncoupled NRCs became less negative but they remained spontaneously active (Fig. 5). Uncoupling also restored action potential shape of NRCs to that observed in single cells. After withdrawal of halothane, gap junctional coupling was restored and the initial synchronous activity reappeared while membrane potentials of both ADC and NRC returned to values as recorded before exposure to halothane (Fig. 5). Upstroke velocities of the triggered ADC action potentials as recorded during the wash-in and wash-out of halothane appeared not significantly different.

3.5. Determinants of triggered activity in ADCs assessed by computer simulation

To validate dependence of triggered activity in ADCs on NRC cluster size and gap junctional conductance, a cluster of spontaneously active cells (SA nodal cells representing NRCs) was coupled to an intrinsically quiescent ventricular cell. Both NRC cluster size and gap junctional coupling conductance between the NRC cluster and the ADC were varied. Fig. 6, A–C, illustrates the outcome of the simulation for a cluster of 10 NRCs. At low coupling, the NRC cluster is beating spontaneously but it cannot pass enough current to the ADC to have it reach threshold (‘pace-but-not-drive’...
At high coupling, the spontaneous activity of the NRC cluster becomes suppressed by the electrotonic interactions with the ADC (‘not-pace’ (NP); Fig. 6C). At intermediate values of coupling conductance, the NRC cluster is beating spontaneously and can pass enough current to the ADC to have it reach threshold (‘pace-and-drive’ (PD); Fig. 6B). Fig. 6D shows the outcome for all combinations of NRC cluster size and NRC–ADC coupling conductance. The asterisks indicate the parameter settings of Fig. 6, A and B. The solid line with closed circles marks the coupling conductance at which beating of small NRC clusters becomes suppressed (transition from PND to NP). The solid line with open circles marks the minimum coupling conductance at which large NRC clusters trigger beating in the ADC (transition from PND to PD). The solid line with closed squares marks the transition from PD to NP that can occur for NRC clusters of intermediate size when electrotonic interactions become too strong. For clusters comprising 7 or 8 cells, an additional transition (from NP to PD), near 6.5 nS, is observed, which is related to the time course of the junctional current. Above 6.5 nS, the ADC approaches threshold more rapidly and the electrotonic load of the ADC is removed (by reaching threshold and firing an action potential) just before spontaneous activity of the NRC cluster ceases, as occurs below 6.5 nS.

3.6. Computer simulations show a vulnerable window for triggered activity of ADCs

In order to validate whether these observations could be extrapolated to a higher level of complexity, additional simulations were performed. In those simulations we mimicked the approach as described by Kehat et al. in which a spontaneously active human ES embryoid body is transplanted in a mature ventricular tissue which is activated only at a low frequency due to a chronic AV-block [6]. In our simulation, a cluster of spontaneously active cells (SA nodal cells representing NRCs) was incorporated into a two-dimensional sheet of intrinsically quiescent ventricular cells. NRC-cluster size was varied, either using a small cluster of 50 cells or a large cluster of 100 cells. Gap junctional conductance between the NRC cluster and surrounding ventricular cells was varied also and set to 10, 20 or 40 nS. If uncoupled (Fig. 7A), the NRC cluster beats spontaneously at a frequency of 2.6 Hz, and surrounding ventricular cells are quiescent with a stable resting membrane potential of −86 mV. At low coupling conductance (Fig. 7B), junctional current flows from the NRC cluster to the surrounding cells, resulting in a decrease in beating frequency of the NRC cluster and subthreshold depolarizations in the surrounding ventricular cells. The large cluster is less affected by the electrotonic interactions with the surrounding myocardium than the small cluster, as demonstrated by a smaller decrease in beating frequency (from 2.6 to 2.1 Hz for the large cluster versus 1.6 Hz for the small cluster), and is able to supply more current to neighbouring cells, as demonstrated by the larger amplitude of the subthreshold depolarizations (from

Fig. 6. Simulated electrical activity of an NRC cluster coupled to an ADC. A–C: Electrical activity of a cluster of 10 NRCs coupled to an ADC with a gap junctional conductance of (A) 5 nS, (B) 10nS, or (C) 20 nS. D: Effect of NRC cluster size (abscissa) and NRC–ADC coupling conductance (ordinate) on electrical activity. Lines with symbols separate regions where (1) the NRC cluster beats spontaneously, but does not trigger beating of the ADC (‘pace-but-not-drive’), (2) spontaneous beating of the cluster is suppressed by the ADC (‘not-pace’), or (3) the cluster beats spontaneously and triggers beating of the ADC (‘pace-and-drive’).
−86 mV to −68 mV for the large cluster versus −71 mV for the small cluster). At moderate coupling conductance, spontaneous activity is suppressed in the small cluster (Fig. 7C, left), but the large cluster continues to beat at a frequency of 1.6 Hz, and is able to supply enough current to the surrounding myocardium to reach threshold and generate triggered activity (Fig. 7C, right). At high coupling conductance, spontaneous activity is suppressed in both clusters (Fig. 7D). These simulations confirm that also at a level of increased complexity, a window of gap junctional conductance values exist that allow a sufficiently large cluster of spontaneously active cells to generate triggered activity in the surrounding ventricular myocardium.

4. Discussion

This study shows: (1) ADCs in culture remain quiescent and largely retain their morphology and electrical phenotype. (2) In culture with spontaneously active NRCs, de novo generated gap junctions between ADCs and NRCs facilitate functional coupling within two days as shown by electrical conductance and dye transfer. (3) Electrical conductance between homotypic cell pairs is much larger than that between heterotypic cell pairs. (4) Small clusters of spontaneously active NRCs are able to trigger a poorly electrically coupled ADC to beat in synchrony. (5) Triggering occurs within a delicate window of gap junctional conductance and is further influenced by the size of the coupled cell-cluster.

4.1. Role of cell-to-cell coupling

Electrical activity of the heart as initiated within the SA node depends on a delicate interplay between spontaneous electrical impulse formation and propagation. The morphological organization of the SA node supports the generated impulse to be conducted out of the node in order to activate the connected atrial working myocardium [1]. The low level of electrical coupling between myocytes within the node is a major determinant of proper pacemaker function since it prevents clamping of the small SA node by the surrounding atrial mass. Differences in expression level and distribution of gap junction channels explain the differential degree in coupling.

The delicate balance of the coupling between spontaneously active cardiomyocytes and surrounding quiescent cells may also play a role in arrhythmogenicity of cell transplantation. Multiple approaches focus on the regeneration of compromised hearts by means of transplantation strategies using in vitro generated donor cells. As mentioned, these cells can originate from various sources. Although completely different in origin, such cells exhibit common electrophysiological features in that they are spontaneously active (SDCs, NRCs) or can be stimulated to elicit undesired electrical activity (skeletal myoblasts). Furthermore, these cells express gap junction proteins at a low level and in a distribution pattern comparable to nodal cells. Those features suggest that such cells might harbour the ability to induce pacemaking activity which was experimentally confirmed in a study by Kehat et al. [6].

Not much is known about the actual electrical interaction between engrafted immature cardiomyocytes and mature host cardiomyocytes. Measuring this interaction in vivo is experimentally complicated if not impossible. Although functional coupling between donor myocytes and the recipient environment has been suggested by the propagation of calcium waves [24], this does not allow determination of the degree of electrical communication. NRCs exhibit an important prerequisite for impulse formation: the absence of a stable resting membrane potential and a slow diastolic depolarization which triggers spontaneous action potential formation. On the other hand, during the first 3 days after isolation cultured ADCs retain their morphology and electrical phenotype. In culture, ADCs and NRCs rapidly (within 48 h) developed gap junctional communication as shown by synchronous beating, dye transfer and electrical coupling. Measurements of electrical conductance between the heterologous cell-pairs revealed that the level of coupling was rather poor. Because of a very high amino acid homology of Cx43 proteins in general [25], this low conductance is unlikely to result from species differences. In our model no docking problems have to be expected since rat Cx43 and canine Cx43 showed an overall identity of 97.1%, with only one residue differing in the extracellular domain (data not shown). More likely, low conductance results from the de novo formation of gap junctions as suggested by the pattern of Cx43 labelling at the interface between pseudopodia-like extensions of the ADC and connected NRCs. Theoretically, coupling could increase upon aging of the cultures. Because it appeared that after 3 days of culture time ADCs changed both structurally (disorganized contractile apparatus, increased membrane capacitance, lateralization of junctional proteins) and electrically (decrease of AP upstroke velocity, trend to decreased $I_{\text{K1}}$), we were restricted to compare the interaction between cultured ADCs and NRCs only during the first 3 days.

4.2. Balance between cluster size and coupling

Most striking, this study shows that pacing of the ADCs is dependent on spontaneous activity of the NRCs, NRC cluster size and low coupling conductance between ADC and NRCs. This relation depicts a pro-arrhythmic source-sink effect which partially can be explained by the low coupling conductance between ADC and NRCs, and the large difference in cell membrane capacitance (ADC: 184±9.7 pF, NRC: 19.6±1.4 pF). Experimental uncoupling of the ADC–NRC interaction clearly demonstrates that: (1) action potential formation of ADCs is triggered by the adjoining NRC cluster, (2) de novo formed gap junctions are responsible for pacing of mature ADCs by immature
NRCs, (3) ADC–NRC syncytia display averaged electrical characteristics of the individual cell types.

Our computer simulations underline that this delicate source–sink relation between NRCs and ADCs in co-culture is favouring pro-arrhythmia. The parameter space diagram of Fig. 6D illustrates the interaction between intrinsic automaticity and intercellular coupling and reflects a generic behaviour that occurs regardless of specific ionic mechanisms.
as recently set out by Pumir et al. (their Fig. 7) [26]. For the experimentally observed NRC–ADC coupling conductance of \(\approx 10 \text{nS}\), our simulations predict two possible outcomes: suppression of spontaneous activity for clusters up to 7 cells and triggered beating of the ADC for clusters of 8 or more cells. The quantitative difference from our experimental results, where triggered activity was already observed for clusters of three of more cells, can be explained by the smaller ‘depolarization reserve’ of our model NRC (only calcium current system available) compared to real NRCs (both calcium and sodium current systems available) as well as the more negative resting potential of our model ADC. Interestingly, our simulations demonstrate that this observation can be extrapolated to the tissue level: a relatively small amount of spontaneously active cells with a moderate level of electrical coupling can also drive an intrinsically quiescent sheet of ventricular tissue.

4.3. Possible consequences for cell transplantation

The low conductance between NRCs and ADCs in coculture might reflect a possible outcome of stem cell transplantation. The \textit{in vitro} interaction shown in this study demonstrated that interactions between mature and immature myocytes arise at the former ID of the mature cell. \textit{In vivo}, it remains open whether a comparable plasticity of the mature ID region is also present to facilitate a \textit{de novo} interaction. Apart from a compromised electrical coupling, the aspect of cluster size in relation to arrhythmogenesis is relevant to the field of stem cell transplantation. The dominant form of stem cell delivery is to inject cells intramurally into the myocardium. This is bound to increase the likelihood of settlement of clumps of SDCs in the injected areas. In hearts where the intrinsic pacing rate of the transplanted cells exceeds the endogenous frequency, such clumps could become an arrhythmogenic substrate when electrical integration with the host myocardium starts to develop giving rise to a low coupling conductance. The fact that injection of embryoid bodies initiates ectopic pacemaking in porcine hearts [27] probably involves both the aspect of cluster size and a low effective coupling to the host myocardium.

4.4. Limitations of the study

In this study we have evaluated the underlying mechanism through which spontaneously active cardiomyocytes are able to trigger intrinsically quiescent adult cardiomyocytes. As source of spontaneously active cardiomyocytes we used neonatal rat ventricular myocytes. These cells partly resemble SDCs in that both cell types display spontaneous activity and have a cell size and coupling characteristics aberrant from adult ventricular myocytes. Whether a broader comparison between SDCs and NRCs allows a stronger implication for stem cell therapy requires additional experimental evidence.

We were unable to study the interaction between a cluster of spontaneously active NRCs and a sheet of cultured ADCs in an \textit{in vitro} co-culture of NRCs and ADCs. The reason is that ADCs cannot be cultured in monolayers without losing their endogenous morphology and electrophysiological characteristics. The latter aspect was shown to change after 3 days of culture time which limited the time period in which experiments could be conducted.

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References