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Cardiac progenitor cells engineered with Pim-1 (CPCeP) develop cardiac phenotypic electrophysiological properties as they are co-cultured with neonatal myocytes

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Abstract

Stem cell transplantation has been successfully used for amelioration of cardiomyopathic injury using adult cardiac progenitor cells (CPC). Engineering of mouse CPC with the human serine/threonine kinase Pim-1 (CPCeP) enhances regeneration and cell survival *in vivo*, but it is unknown if such apparent lineage commitment is associated with maturation of electrophysiological properties and excitation–contraction coupling. This study aims to determine electrophysiology and Ca^{2+} -handling properties of CPCeP using neonatal rat cardiomyocyte (NRCM) co-culture to promote cardiomyocyte lineage commitment. Measurements of membrane capacitance, dye transfer, expression of connexin 43 (Cx43), and transmission of ionic currents (I_{Ca} , I_{Na}) from one cell to the next suggest that a subset of co-cultured CPCeP and NRCM becomes connected *via* gap junctions. Unlike NRCM, CPCeP had no significant I_{Na} , but expressed nifedipine-sensitive I_{Ca} that could be measured more consistently with Ba^{2+} as permeant ion using ramp-clamp protocols than with Ca^{2+} and step-depolarization protocols. The magnitude of I_{Ca} in CPCeP increased during culture (4–7 days *vs.* 1–3 days) and was larger in co-cultures with NRCM and with NRCM-conditioned medium, than in mono-cultured CPCeP. I_{Ca} was virtually absent in CPC without engineered expression of Pim-1. Caffeine and KCl-activated Ca^{2+} -transients were significantly present in co-cultured CPCeP, but smaller than in NRCM. Conversely, ATP-induced (IP_3 -mediated) Ca^{2+} transients were larger in CPCeP than in NRCM. I_{NCX} and I_{ATP} were expressed in equivalent densities in CPCeP and NRCM. These *in vitro* studies suggest that CPCeP in co-culture with NRCM: a) develop I_{Ca} current and Ca^{2+} signaling consistent with cardiac lineage, b) form electrical connections *via* Cx43 gap junctions, and c) respond to paracrine signals from NRCM. These properties may be essential for durable and functional myocardial regeneration under *in vivo* conditions.

Keywords

Stem cell; Calcium; Ion channels; Electromechanical coupling; NCX; Pim-1

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Disclosure statement

None.

1. Introduction

Regeneration and repair of damaged myocardium has been attempted using multiple cell types including embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), fetal myocytes, skeletal myoblasts, endothelial progenitor cells and bone-marrow stem cells [1]. Recent advances in cell biology of adult cardiac stem cells (CSC) in murine, canine and human myocardium have revealed an endogenous reservoir of multipotent cardiac progenitor cells (CPC) that are c-kit⁺ and/or Sca-1⁺, self-renewing, clonogenic, and appear to have promising potential for regeneration of the damaged/failed myocardium [2–6]. CSC in long term culture exhibit stem cell-like properties of single-cell derived clone formations with the potential to differentiate into the three main lineages: cardiomyocytes, smooth muscle and endothelial cells *in vitro* that also promote increased cardiomyocyte survival in co-culture [7]. However, the extent to which adoptively transferred CPC acquire electrophysiological and mechanical coupling to host cardiomyocytes remains controversial [8,9]. Long term persistence and functional integration of adoptively transferred cells remains a significant barrier to efficacious CPC-based therapy, but genetic modification of CPC to express Pim-1 kinase (CPCeP) appears to improve regeneration, remodeling, and hemodynamic function of the infarcted myocardium up to 32 weeks in hearts of recipient mice [10]. The enhanced expression of Pim-1 was found to stabilize proliferative proteins downstream of nuclear Akt signaling and increase proliferation and metabolic rates in CPCeP when compared with CPC. The long-term engraftment of CPCeP may include electromechanical coupling essential to the regenerative process, but the electrophysiological and Ca²⁺-signaling properties of CPCeP have not been characterized.

Cardiac embryonic and postnatal development involves a programmed increased expression of Ca²⁺ release (ryanodine receptors, RyR, IP₃R), and Ca²⁺ uptake proteins (SERCA2a, phospholamban) of the sarcoplasmic reticulum (SR), and L-type Ca²⁺ channels (DHPR) of the surface membrane, whereas Na⁺–Ca²⁺ exchanger (NCX) expression levels remain constant or decrease slightly [11]. Ca²⁺ current density increases from fetal development to birth while SR maturation lags behind [12], but fully developed Ca²⁺ stores gated by Ca²⁺-induced Ca²⁺ release (CICR) are present in 1–2 day old isolated neonatal cardiomyocytes [13]. Gating of Ca²⁺ release from RyR by Ca²⁺ influx through DHPR evolves early in developing myocytes and becomes the primary trigger of CICR from the SR in adult cardiomyocytes [14]. During this early developmental period there also appears to be a shift in the expression levels of IP₃-gated Ca²⁺ stores to RyR-gated release mechanisms [14].

In comparison, significant differences are evident when comparing electrophysiological and Ca²⁺ signaling events for various stem cell types. For instance, undifferentiated human ESCs and human ESC-derived cardiomyocytes exhibit rapidly activating delayed rectifier potassium current (I_{Kr}), a hyperpolarizing current (I_f) and an L-type calcium current (I_{Ca}) [15], whereas undifferentiated human iPSCs lack I_{KCa} , I_f , I_{Na} and I_{Ca} currents [16], but instead exhibit TEA-sensitive delayed rectifier K⁺ currents (I_{KDR}). Similarly, while I_{KCa} , I_{Kir} , and I_{Cl} were recorded in undifferentiated mouse bone-marrow mesenchymal stem cells [17], only outward currents were present in undifferentiated mouse bone-marrow c-kit⁺ cells [18]. Yet others have found functional I_{KDR} , I_{Kir} and $I_{Cl,vol}$ in mouse undifferentiated cardiac c-kit⁺ stem/progenitor cells [5]. When c-kit⁺ cells were co-cultured with mouse neonatal cardiac myocytes for 7 days, though cardiac markers for voltage-gated Na⁺ and Ca²⁺ channels appeared to be expressed, electrophysiologically functioning Na⁺ and Ca²⁺ currents or functional gap-junctions were absent [18]. Considering such marked electrophysiological difference of various stem cells at different stages of differentiation [19] it may be pertinent to evaluate the factors that may cause them to emerge from a developmentally immature state. These differences may not only reflect inherent differences

in the cell types, but also the means used to guide their differentiation and investigate their properties.

Since it has been already reported that introduction of CPCeP in injured myocardium leads to significant recovery of function in whole animal hearts possibly mediated by direct electromechanical coupling between the two sets of cells or through paracrine effects of introduced cells (such as insulin-like growth factor, IGF-1, or vascular endothelial growth factor [7], or both), we undertook a study to determine the functional expression of ionic channels and Ca^{2+} -signaling pathways in a controlled *in vitro* co-culture of CPCeP and neonatal rat cardiac myocytes (NRCMs) where the extent to which electrophysiological integration between the two sets of cells occurring within first 7 days of co-culturing was quantified. We found significant electrophysiological integration of Pim1 expressing cells with neonatal rat myocytes, allowing increased expression of calcium current, RyR- and IP_3R -gated Ca^{2+} stores, and Cx43 expression in Pim1 expressing cells either when these were co-cultured with NRCM, or were cultured in media conditioned by growing NRCM.

2. Materials and methods

2.1. Cell culture

Four- to six-day-old neonatal rats were decapitated, the chest cavities opened, hearts excised, and the main vessels and atria removed. The ventricles were minced with a razor blade and incubated in Hank's Balanced Salt Solution (HBSS, Invitrogen) with trypsin (50 $\mu\text{g}/\text{ml}$) for 14–16 h at 4 °C. The digestion was then arrested by exposure to trypsin inhibitor (200 $\mu\text{g}/\text{ml}$) for 20 min. Collagenase (100 U/ml) was used for 30 min to isolate single NRCM, which were then filtered and centrifuged at 1000 rpm for 3 min, re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) with 1% penicillin–streptomycin and 1% non-essential amino acids, plated on 100-mm dishes and placed in the incubator for 60 min to eliminate fibroblasts. NRCM overall viability was ~80%. Isolated single NRCM were plated onto non-treated glass cover slips and used for electrophysiological experiments. Another group of NRCM was suspended in a concentration of 10^5 cell/ml in DMEM with 10% FBS and incubated for 7 days at 37 °C to produce the precursor for “conditioned medium”. At this time the supernatant was extracted, centrifuged, and stored at –20 °C for later treatment of one group of mono-cultured CPCeP.

As previously reported [10], CPCeP were derived from adult mouse CPC that were genetically modified to express enhanced green fluorescent protein (eGFP) and human Pim-1 kinase. Expression of the human Pim-1 gene was verified by immunoblot of cell lysates for Pim-1 protein, showing substantial elevation of the transgene expression relative to eGFP-expressing controls [10]. The CPCeP were passaged once a week and cultured in DMEM-F12 (Invitrogen) containing 10% FBS in the presence of LIF, ITS, EGF and bFGF at 37 °C. Experiments were performed on CPCeP from the 1st to 3rd passages. The percentage of CPCeP with a clear eGFP-positive signal was 70–80%. After 7 days in culture, CPCeP were harvested using trypsin, re-suspended, and plated onto cover slips and cultured alone or with neonatal rat cardiomyocytes using standard medium (100% DMEM-F12). A second group of re-suspended CPCeP was cultured for 1–7 days in conditioned medium consisting of 75% DMEM-F12 medium and 25% of the above-mentioned stored extract. In this case the initial cell density was not more than 10^3 cell/ml to allow the CPCeP to proliferate for up to 7 days without overcrowding or confluency or requiring replacement of the conditioned medium.

2.2. Electrophysiological recordings

Cells were bathed in Tyrode's solution containing (in mM): 137 NaCl, 5.4 KCl, 2 CaCl₂, 10 HEPES, 1 MgCl₂, and 10 Glucose (pH 7.4 with NaOH). Whole-cell patch-clamp recordings were performed using a Dagan voltage-clamp amplifier and pClamp software. Borosilicate patch pipettes were prepared using a horizontal pipette puller (Model P-87, Sutter Instruments, CA). The pipettes had a resistance of 3–5 MΩ when filled with one of two solutions containing (Sol-1, with weak Ca²⁺-buffering capacity, in mM): 15 NaCl, 100 CsCl, 20 TEACl, 0.5 MgCl₂, 0.1 Ca₂Cl, 0.2 EGTA, 10 HEPES, 10 Glucose, 5 MgATP and 0.1 cAMP (pH 7.15 with CsOH; measured osmolality: 290 mOsm) or (Sol-2, with stronger Ca²⁺-buffering capacity, in mM): 15 NaCl, 94 CsCl, 10 TEACl, 6.2 Ca₂Cl, 14 EGTA, 10 HEPES, 10 Glucose, 5 MgATP and 0.1 cAMP (pH 7.2 with CsOH; 300 mOsm). The intracellular free Ca²⁺ concentrations ([Ca²⁺]_i) were calculated to be 173 nM for Sol-1, and 100 nM, for Sol-2.

L-type Ca²⁺ current (I_{Ca}), Na⁺ current (I_{Na}), Na⁺–Ca²⁺ exchanger current (I_{NCX}), and ATP-activated current (I_{ATP}) were measured using the whole-cell voltage-clamp technique. These components of membrane current were surveyed using voltage-clamp protocols consisting of sequences of step-wise depolarization (I_{Ca} , I_{Na} ; Figs. 2B, 3 and 4), depolarizing ramps (I_{Ca} , I_{Na} ; Figs. 2C and 5), re-polarizing ramps (I_{NCX} ; Fig. 6), and application of ATP at a fixed holding potential (I_{ATP} , –80 mV; Fig. 8). The step-protocols were executed with Sol-1 using depolarizing pulses of 50–70 ms duration that were incremented by 10 mV from holding potentials that were designed to activate only I_{Ca} (–40 mV) or also I_{Na} (–80 mV). The depolarizing ramp pulses, (ascending from –60 (I_{Ca}) or –80 (I_{Na}) to +80 mV during a 30–60 ms interval) were used in conjunction with elevated Ca²⁺ concentrations ([Ca²⁺]_o = 2–5 mM) or 10 mM Ba²⁺ and nifedipine (10 μM) as a faster and more sensitive way of measuring I_{Ca} in PCPePs. Similarly I_{NCX} was measured with ramps descending from +80 to –120 mV in 200–400 ms using elevated [Ca²⁺]_i (170 nM, Sol-2) to enhance this current, and 5 mM Ni²⁺ as a blocking agent. Drugs and chemicals were applied using an electronically controlled micro-perfusion system which allows rapid drug/chemical application onto single cells in ~20 ms. Thus the existence of I_{ATP} was tested by rapid 1–2 s application of 100 μM ATP at –80 mV.

Cell membrane capacitance (C_m) was determined from the integral of capacitive current transients using pClamp 10 software (Molecular Devices Corporation, CA, USA). The mono- or bi-exponential time course of these current transients was used to assess if a patch-clamped cell was electrically detached or connected electrically to other cells in its immediate vicinity. This issue was also addressed by: a) visual inspection of proximity and synchronized contractions, b) comparison of C_m with estimated areas of cell membrane, c) dye transfer between cells, and d) evaluation of gap junctions by fluorescence imaging of immuno-labeled connexin 43 (Cx43).

All experiments were carried out at room temperature (22–25 °C).

2.3. Dye transfer between cells

Coupling of CPCeP to NRCM through gap junction formation was assessed using a fluorescent dye (Lucifer yellow, 150 μM), which was added to the internal patch pipette solution and dialyzed into individual voltage-clamped CPCeP cells (Fig. 1). I_{Ca} and I_{Na} were simultaneously monitored.

2.4. Fluorescence imaging of immunolabeled Cx43

Immunohistochemical approaches were used to detect Cx43 in cultures of CPCeP and NRCM. Media were removed and cells were rinsed with phosphate-buffered saline (PBS),

and then fixed with 4% paraformaldehyde for 10 min at room temperature. After washing the cells 3 times with PBS, the cells were blocked overnight at 4 °C in 1% bovine serum albumin and 0.1% Triton in PBS. Then the blocking solution was removed, and primary antibody (1:2000 anti Cx43(r) by Sigma) was applied for 2 h at room temperature and an additional 2 h at 4 °C in PBS containing 0.05% Triton. After washing out the cells 3 times with PBS, anti-rabbit Alexa Fluor 555 (1:2000) in PBS was applied for 2 h at room temperature. The secondary antibody dilution was removed and the cover slip was mounted on a slide using a drop of ProLong Gold with DAPI and allowed to cure for 24 h at room temperature and stored at −20 °C. Imaging was performed on a Leica TCS SPS AOBs confocal microscope system using a 488 nm argon ion laser to examine eGFP expression and a 555 nm Helium Neon Laser to examine Cx43 expression. Quantification of Cx43 was done by Adobe Photoshop and ImageJ v1.45 (Fig. 9).

2.5. Two-dimensional confocal Ca²⁺ imaging

Intracellular Ca²⁺ signals of CPCeP and NRCM were measured with the fluorescent Ca²⁺ indicator dye Fluo-4 AM (20 μM) and imaged using a Noran Odyssey XL rapid two-dimensional laser scanning confocal microscope (Noran Instruments, Madison, WI) attached to a Zeiss Axiovert TV135 inverted microscope with a 63× water-immersion objective lens. The excitation wavelength of the argon ion laser was set to 488 nm and fluorescence emission was measured at wavelengths >515 nm. Cells were imaged at 4–120 frames/s depending upon the experiment. Ca²⁺-dependent fluorescence signals were activated by rapid 1–2 s long “puffs” of ATP (100 μM), caffeine (10 mM) and KCl (100 mM). Images were filtered by 3 pixel × 3 pixel averaging and the calculated fluorescence intensities were expressed as F/F₀, where F is the peak value and F₀ is the average resting fluorescence intensity. Ratiometric confocal images were obtained by dividing the fluorescence at a given time and location (F(x,y,t)) with the Fluo-4 baseline fluorescence at the same location (F₀(x,y)). When used in conjunction with bright field images and images of baseline fluorescence, such ratiometric images (frames 1 and 2 in Figs. 7A–C) facilitated the identification of individual cells as regions of interest (Fig. 7E) where the local amplitudes and kinetics of the Ca²⁺ signals showed a high degree of uniformity.

2.6. Solutions and chemicals

Solutions with ATP (100 μM), caffeine (10 mM), and KCl (100 mM, replacing equimolar concentrations of NaCl) were prepared using Tyrode's solution as a base. Lucifer yellow (150 μM) was dissolved in the internal patch pipette solution for daily use. Nifedipine (10 μM) stock solution was prepared in DMSO and was diluted in the external K⁺-free Tyrode's solution, and Fluo-4 AM (20 μM) was dissolved in DMSO and diluted in the external Tyrode's solution. All drugs and chemicals used in the experiments were prepared daily.

2.7. Statistical analysis

Results are expressed, as mean ± SEM with “n” referring to the number of cells examined. Unpaired student's *t*-test or one way analysis of variance (ANOVA) *post hoc* Newman–Keuls was used to evaluate differences between measurements from more than two different groups. *p* < 0.05 was considered statistically significant and is marked with a single asterisk (*). Higher significant degrees were expressed with two (**, *p* < 0.01), and three (***, *p* < 0.001) asterisks. Figs. 2D and 5C show results of Bernoulli trials. When no positive results were found (*n*₁ = 0) in a total of *n*₂ trials, the shown means and standard deviations were calculated as 1/(*n*₂ + 2).

3. Results

3.1. Survey of structure and currents of cultured CPCeP

We compared the ionic currents in voltage-clamped CPCeP that were maintained in culture for 1 to 7 days either alone or together with NRCM. This approach required evaluation of the changes that reflect altered expression of ion channels in the CPCeP and the co-cultured NRCM. Fig. 1 illustrates the changing morphology of the CPCeP and their electrical connection to NRCM. CPCeP were spherically shaped with 10–15 μm in diameters when initially plated, but generally showed spindle-shaped outgrowth and proliferation when used 2 to 7 days later (Figs. 1A, B). After 7 days in culture, the CPCeP lost their spherical shape, and were seen as large (~100–150 μm) multilateral flat cells with very long dendrite-like outgrowths. For electrophysiological studies we generally selected the newly growing CPCeP with positive expression of eGFP.

Several criteria were used to determine the degree of physical coupling and electrical communication between CPCeP and NRCM in co-cultures. CPCeP growing in proximity of neonatal cardiomyocytes (Fig. 1C) were mechanically and synchronously distorted by contracting myocytes, an indication that cells were physically connected to each other. Evidence for electrical connectivity was obtained from the shape of the capacitive currents that were elicited by small (10–20 mV) step depolarizations. Unattached single cells produced brief capacitive currents corresponding, on average, to membrane capacitances (C_m) of 52.7 ± 2.3 pF ($n = 219$) for CPCeP and 52.6 ± 10.2 ($n = 15$) for NRCM (Fig. 1E). In contrast CPCeP that were attached to NRCM produced bi-exponentially decaying capacitive currents with a total integral corresponding to 106.9 ± 14.8 pF ($n = 25$) (Fig. 1D), about double the values of the unattached cells. The “transmission” of current from a voltage-clamped CPCeP to its attached NRCM in measurements of cell capacitance was particularly striking in cases where electrical contact was lost due to myocyte injury in the course of an experiment (Fig. 1D). The measured C_m of the relatively flat cells was in rough agreement with cell size estimates based on Cole's constant and twice the visible area of the cells (e.g. $1 \mu\text{F}/\text{cm}^2 \cdot 25 \mu\text{m} \cdot 100 \mu\text{m} \cdot 2 = 40$ pF). Coupling between cells was also detected by the diffusion of Lucifer yellow. In cells with typical electrical coupling, we observed that this fluorescent marker first penetrated to the voltage-clamped CPCeP cell, and then to adjacent NRCM with a delay of ~2 min (Figs. 1F, G, $n = 6$), thereby confirming cytoplasmic continuity between the cells. These observations suggest that recorded membrane currents in some cases may originate not only from the voltage-clamped cell itself, but also from connected cells that may be less effectively clamped due to cell-to-cell resistance. Such currents we call “transmitted” or “reflected” currents.

As illustrated in Fig. 2, we surveyed the presence of different ionic currents in voltage-clamped CPCeP that were either cultured alone, or co-cultured with NRCM. Using step-clamp protocols (Fig. 2B), we found that separately cultured CPCeP did not produce detectable Ca^{2+} currents, I_{Ca} , or Na^+ currents, I_{Na} (Fig. 2D). Surprisingly they produced significant-nifedipine sensitive I_{Ca} (Fig. 2C) when examined with ascending ramps of 30–60 ms duration from –60 to +80 mV in solutions containing 2–5 mM Ca^{2+} . With this strategy we measured significant I_{Ca} (>0.4 pA/pF) in 23 out of 64 monocultured CPCeP (36%), but found no indication of I_{Na} even when the holding potential preceding the ramp potential was altered to –80 mV. Although the ramp-clamp protocol improved upon measurements of I_{Ca} induced by repetitive step depolarizations protocol, the current continued to run down rapidly. Current through the Ca^{2+} channels was measured more reliably with 10 mM Ba^{2+} as charge carrier. This situation was significantly different when CPCePs were voltage-clamped in co-cultures with NRCM. In this case the step-clamp protocol produced significant I_{Ca} in 8 out of 64 cells, and I_{Na} in 6 out of 61 cells while the ramp protocol activated I_{Ba} in 34 out of 68 cells (50%). In contrast ATP activated currents, I_{ATP} , and Na^+ –

Ca^{2+} exchanger currents were present in 30–40% of voltage-clamped CPCeP independent of culture conditions.

Considering the evidence for electrical coupling between CPCeP and co-cultured NRCM (Fig. 1) we examined the possibility that the I_{Ca} and I_{Na} that, under these conditions, were produced by the step-clamp protocol in ~10% of the CPCeP, might be “transmitted” from the NRCM.

3.2. Calcium currents in CPCeP

Based on the finding that only few co-cultured CPCeP produced I_{Ca} (12.5%) and I_{Na} (10%) in response to *step* depolarizations, we compared the characteristics of these currents with those obtained from NRCM.

Fig. 3 compares the time course (panel A), delay in the activation (panel B), voltage-dependence (panel C) and maximal amplitude of I_{Ca} (panel D) measured from: 1) co-cultured CPCeP attached to NRCM, 2) NRCM in mono-cultures, and 3) co-cultured NRCM attached to CPCeP. The voltage-dependence of I_{Ca} was the same in all three cases: I_{Ca} activated at -40 mV, peaked around 0 to $+10$ mV and reversed around $+40$ mV (Fig. 3C). Calcium currents in CPCeP attached to NRCM averaged about 150 ± 22 pA, as compared to 128 ± 26 pA and 251 ± 66 pA in NRCM mono-cultures or in co-culture of myocytes attached to CPCeP, respectively. However, current densities (pA/pF) of I_{Ca} normalized according to membrane capacitance, were significantly lower in CPCeP attached to NRCM (1.13 ± 0.20 pA/pF) than those recorded in NRCM monocultures (3.36 ± 0.70 pA/pF) or NRCM attached to CPCeP (4.02 ± 0.18 pA/pF, $p < 0.01$) (Figs. 3C and D).

Fig. 3A, (sample traces) illustrates another interesting property of membrane currents recorded from CPCeP attached to NRCM: capacitive transients were long lasting (~ 10 ms); activation of I_{Ca} was delayed significantly; and the time-to-peak of I_{Ca} was significantly longer (22.7 ± 5.1 ms) for CPCeP attached to NRCM than for unattached NRCM (5.6 ± 0.7 ms) or NRCM attached to CPCeP (4.9 ± 0.4 ms) (Fig. 3B). Fig. 4 illustrates similar effects for I_{Na} . The average peak value of I_{Na} , measured with step depolarization from -80 to -30 mV was 2660 ± 510 pA in CPCeP attached to NRCM compared to 5200 ± 610 pA in isolated NRCM and 4980 ± 1850 pA in NRCM attached to CPCeP. The corresponding current densities of I_{Na} were 29 ± 6 pA/pF in CPCeP attached to NRCM, 151 ± 24 pA/pF in NRCM monocultures, and 64 ± 23 pA/pF in NRCM attached to CPCeP (Figs. 4C, D). The voltage-dependence, including the apparent reversal potential (30 mV) was the same in all preparations. Time-to-peak of I_{Na} recorded from CPCeP attached to NRCM (5.1 ± 0.9 ms) was significantly longer than the values recorded in NRCM monocultures (3.1 ± 0.2 ms) or in NRCM attached to CPCeP (2.43 ± 0.17 ms) (Fig. 4B).

Collectively, these data suggest that a small proportion of CPCeP ($\sim 10\%$) are attached electrically to NRCM within the first 7 days of co-culture conditions and that I_{Ca} and I_{Na} measured from the CPCeP under these conditions include a major component that is transmitted from the NRCM into CPCeP.

Since we suspected that I_{Ca} and I_{Na} in CPCeP were running down rapidly in response to multiple step-clamp pulses and thereby failing to activate detectable I_{Ca} and I_{Na} in mono-cultures of CPCeP (Fig. 2D), we used the ramp-clamp protocol (Fig. 2C) to evaluate the effects of co-cultured NRCM on I_{Ca} and I_{Na} (Fig. 5). Even though the ramp-clamp protocol failed to reveal detectable I_{Na} in unattached CPCeP, it often activated quantifiable nifedipine-sensitive Ca^{2+} -channel currents in them when 10 mM Ba^{2+} was used as charge carrier through the channel.

We also examined whether the effects of NRCM on CPCeP required direct presence of myocytes in the co-culture or whether the effect was transmitted by paracrine factors. To test the paracrine effect we collected the media from 7 day cultures of NRCM and then cultured the CPCeP in these media. The single CPCeP that were selected for quantification of currents generated by ramp-clamp depolarization were round in shape and not connected to other cells. Fig. 5 shows the amplitude distributions for “responsive” cells with significant I_{Ca} (>0.4 pA/pF; panel A), the average values of these distributions (panel B), and fraction of cells that produced these responses (panel C). For comparison panels B and D include values obtained in parallel experiments with cardiac progenitor cells (CPC, red data points) without engineered expression of Pim-1 kinase. In CPCeP incubated in regular culture media for 4–7 days, I_{Ca} was larger (1.61 ± 0.36 pA/pF, $n = 8$) than that measured in 1–3 day cultures (0.82 ± 0.12 pA/pF, $n = 13$, $p < 0.05$; panel B, open black circles). Co-cultures of CPCeP with NRCM (filled black circles) and monocultures of CPCeP in conditioned media (filled black triangles) generally had larger I_{Ca} and showed a similar increase in magnitude with increasing time in culture. In contrast, panel C suggests that growing CPCeP in regular culture medium for longer times decreased the fraction of cells with significant I_{Ca} (15 out of 32 cells or 47% after 1–3 days vs. 8 out of 32 cells or 25% after 4–7 days). Although CPCeP in co-culture with NRCM initially had more responsive cells (21/29 = 72%) in time this fraction declined similarly (to 13/39 = 33%). The reverse trend was observed with CPCeP in conditioned medium where the fraction of responsive cells in time increased from 50 to 74%. CPC without Pim-1 kinase produced only minimal I_{Ca} in brief monocultures (0.53 ± 0.30 pA/pF in 4 out of 32 cells) and no detectable I_{Ca} when co-cultured with NRCM (24 cells tested).

Collectively, these results indicate that the expression of Ca^{2+} channels in CPCeP is enhanced compared to CPC and is amplified in the course of culture with NRCM or in NRCM-conditioned media.

3.3. NCX expression is comparable between CPCeP and NRCM

Functional expression of NCX (I_{NCX}) was compared between CPCeP, NRCM, and in CPCeP cells attached to NRCM using a descending ramp-clamp protocol from 80 to -120 mV (Fig. 6B, inset). We found a Ni^{2+} -sensitive NCX current in CPCeP similar to that of NRCM. There were no significant differences in the level of expression of I_{NCX} between NRCM, CPCeP, and CPCeP cells attached to NRCM (Fig. 6C).

3.4. Ca^{2+} -signaling in co-cultures of CPCeP and NRCM

The expression of multiple Ca^{2+} signaling pathways was assessed in co-cultures of CPCeP and NRCM using agonists that are known to activate either the Ca^{2+} channel/RyR-signaling pathway (10 mM caffeine, 100 mM KCl) or IP_3 -signaling pathway (100 μ M ATP). Ca^{2+} transients in CPCeP and NRCM were measured using confocal fluorescence imaging of the Ca^{2+} -indicator Fluo-4 AM. NRCM and CPCeP responded differently to KCl, ATP and caffeine. While Ca^{2+} -transients activated by caffeine and KCl were larger in NRCM, those activated by ATP were generally larger in CPCeP (Fig. 7F).

The profile of caffeine-induced Ca^{2+} transients in CPCeP differed greatly from that of NRCM. While caffeine-induced intracellular Ca^{2+} concentrations rose rapidly in NRCM (red and orange traces and regions, Figs. 7A, E), the rise in Ca^{2+} was slow and delayed in CPCeP (purple, dark blue and olive) or absent (light blue and green). Depolarization with KCl produced responses with similarly graded amplitudes (Fig. 7B). Although the delays in this case were much briefer, recording at a frame rate of 30 Hz, it was clear that the KCl-induced responses in CPCeP developed at a later time than in the NRCM. The decay of the caffeine- and KCl-induced Ca^{2+} transients was very slow in CPCeP, so that after 2–3 s, the

Ca^{2+} -levels in these cells were larger than in the more rapidly relaxing NRCM (Figs. 7A, B). In detailed frame-by-frame analysis, we probed whether the Ca^{2+} signals generated in NRCM might spread as waves from NRCM to adjoining sprouting cellular processes of CPCeP. We found no support for this idea. These results are consistent with the idea that delayed, slowly relaxing caffeine- and KCl-induced responses arise directly from CPCeP, rather than being transmitted from NRCM.

Ca^{2+} transients induced by ATP in CPCeP were also significantly different in profile than those recorded from NRCM. CPCeP responded more slowly to ATP but unlike the NRCM signals these were maintained for several seconds (Fig. 7C). Repetitive application of ATP in CPCeP resulted often in smaller Ca^{2+} transients.

All electrophysiological and confocal imaging experiments that were carried out in CPCeP and NRCM were also carried out using co-cultures of CPCeP and adult feline cardiomyocyte without revealing any noticeable qualitative differences (data not shown).

3.5. ATP-activated currents (I_{ATP})

In co-cultures, ATP-activated currents were recorded both in NRCM and CPCeP. In voltage-clamped CPCeP the magnitude of I_{ATP} was comparable (~ 0.4 pA/pF, Fig. 8B) whether the current was recorded from single unattached cells or those in contact with NRCM. In isolated voltage-clamped NRCM I_{ATP} was significantly larger (~ 1.2 pA/pF). Similar to the ATP-induced Ca^{2+} transients, I_{ATP} in NRCM activated and declined more rapidly than in CPCeP (*cf.* Figs. 8A and 7C), suggesting possible contribution also from I_{NCX} .

3.6. Expression of connexin 43

To explore the nature of the coupling between co-cultured CPCeP and NRCM, we used confocal fluorescence microscopy of fixed cell-culture preparations to examine the distribution of immunolabeled Cx43. Fig. 9 illustrates results from mono-cultures (panels A–D) and co-cultures of CPCeP (panels E–G). The fluorescence from Cx43 is shown in red and the fluorescence from eGFP-expressing CPCeP in green. In addition to showing the general morphology of the CPCeP (Figs. 9A, E), the images contained distributed punctuate aggregates of Cx43. To quantify the numbers of Cx43 particles we focused on the sparser regions of the cultures and identified individual CPCeP that had a single nucleus, a well defined outline in the eGFP image, and made relatively few contacts with surrounding cells. Within such outlines (Figs. 9B, F), the number of particles increased from 28 ± 6 ($n = 12$) per cell in mono-cultures of CPCeP to 67 ± 5 ($n = 15$) per cell in CPCeP co-cultured with NRCM. Using identical methods of analysis, the respective sizes of the Cx43 particles were similar ($0.40 \pm 0.05 \mu\text{m}^2$, $n = 12$ vs. $0.47 \pm 0.03 \mu\text{m}^2$, $n = 15$). The Cx43 particles were often seen near the perimeter of cells and in some cases were concentrated in regions where cells appeared to be in contact with each other. Thus, Fig. 9C (arrow) shows that the particles were concentrated at the intersection between two CPCeP with different levels of eGFP expression. Fig. 9D shows a high concentration of Cx43 particles at the interface between a CPCeP (green) and another cell (*, dashed white line) that may correspond to a cardiac progenitor cell without eGFP expression or a contaminating fibroblast. Fig. 9G shows an abundance of Cx43 particles at the interface between a CPCeP (green) and a co-cultured cell with NRCM-morphology. As in Fig. 1F, the NRCM appears to be closely associated with a thin outgrowth from a CPCeP. These observations suggest that CPCeP may communicate with each other and with co-cultured NRCM *via* gap-junctions formed by Cx43.

4. Discussion

Pim-1 continues to be the subject of intensive investigation in the context of cancer biology where the kinase plays diverse roles in cell proliferation, differentiation, and tumorigenesis [20]. More recently, the participation of Pim-1 in the cardiovascular context was initially identified [21,22] and subsequently employed as a genetic interventional strategy to enhance stem cell-mediated myocardial regeneration using CPCs derived from either murine [10] or human heart tissue [23]. In both murine and human CPCeP, enhanced predisposition toward cardiogenic lineage commitment is evident in analysis of mRNA transcript levels of MEF2C, von Willebrand factor, and GATA-6. Recent observations show enhanced asymmetric chromatid segregation and proliferation indicating that Pim-1 has the capacity to promote the expansion of the stem cell population while simultaneously promoting generation of cells destined for lineage commitment [24]. These findings are consistent with studies linking Pim-1 to endothelial and mural cell differentiation [25,26]. The full range of Pim-1 in the cardiomyocyte context continues to be revealed as a novel regulatory molecule with phenotypic effects that partially overlap with, but are in certain respects distinct, from the related upstream nodal Akt kinase that can induce Pim-1 expression [27].

Based on the regenerative myocardial properties of CPCeP [10,23] we hypothesized that these cells might have distinct electrophysiological characteristics. Since it was not feasible to address this issue in the *in vivo* context, we used the co-culture approach *in vitro* to simulate the functional coupling that might occur between CPCeP and cardiomyocytes. The major findings of this report are that CPCeP express I_{Ca} , I_{NCX} , I_{ATP} and a Ca^{2+} -signaling system gated by both IP_3 Rs and RyRs. The expression of Ca^{2+} channels increased during 7 days of culture and was enhanced by co-cultured NRCM and conditioned medium derived from separately cultured NRCM. Electrophysiological communication between CPCeP and NRCM was confirmed by dye-transfer experiments and immunohistochemical evidence for Cx43. These findings are consistent with the idea that CPCeP integrate functionally with myocytes and develop significant Ca^{2+} current and CICR.

4.1. Structural integration of CPCeP with NRCM

Structural considerations of CPCeP are an important part of the analyses presented here. We found that the membrane capacitance of CPCeP was comparable to the value reported for mouse cardiac c-kit⁺ cells [5]. In some CPCeP we recorded a higher membrane capacitance that, on average, equaled the sum of the individual membrane capacitances of CPCeP and NRCM (Fig. 1E). Most likely this arises from attachments formed between CPCeP and NRCM under co-culture conditions (Fig. 1D). Such electrical couplings are consistent not only with long lasting capacitive currents, but also with delayed activation of I_{Ca} and I_{Na} that are transmitted from attached NRCM to voltage-clamped CPCeP (Figs. 3A, B and 4A, B), cell-to-cell dye transfer (Figs. 1E, G), and immunohistochemical determination of Cx43 (Fig. 9). It is well known that gap junctions formed by connexins are essential for cardiac myocyte coupling and electrophysiological integration. Although there are reports that ESC-derived cardiomyocyte and feline cardiomyocytes form gap junctions and establish electrical coupling with cardiomyocyte [28], Lagostena et al. were unable to observe transfer of Alexa Fluor 594, a gap junction permeable dye, in any of the 5 patched undifferentiated mouse bone marrow c-kit⁺ cells injected with this tracer [18]. On the other hand, Ferreira-Martins et al. provide evidence that undifferentiated human CPC express Cx43 gap junction protein [29]. It has been reported that adult ventricular myocytes were connected by gap junctions in intercalated discs for longitudinal connectivity [30] while connexins distribute over the entire surface of neonatal ventricular myocytes in a punctuate pattern [31]. Similarly we found that Cx43 was distributed over the surfaces of both CPCeP and NRCM, but it also appeared to be concentrated in areas of contact between cells (Fig. 9). Our data are consistent with the presence of functional gap junction proteins between CPCeP and NRCM.

4.2. Calcium currents

Appropriate expression of calcium and sodium currents is an essential characteristic of cellular cardiogenic phenotype. Initial attempts in CPCeP to measure I_{Ca} and I_{Na} using step-depolarizing pulse protocols failed to detect significant levels of I_{Na} , or I_{Ca} . The protocol for measuring I_{Ca} was then changed to a ramp protocol. This approach revealed distinct inward going currents that were enhanced by replacing Ca^{2+} with Ba^{2+} and were completely blocked by 10 μM of nifedipine. The ramp protocol most likely protects the patched cells from the run-down effects of multiple depolarizing pulses to positive potentials, while influx of Ba^{2+} inhibits Ca^{2+} -dependent inactivation thus allowing the current to be fully measured.

Mouse bone-marrow c-kit⁺ cells have been reported to lack I_{Na} and I_{Ca} using step depolarization protocols from -70 to $+50$ mV despite expression of cardiac markers for Na^+ and Ca^{2+} channels in 7 day co-cultures [5,18]. This is consistent with our observations with step-depolarization, but not with our ramp-clamp findings. On the other hand, Ca^{2+} currents have been reported in a small portion of bone-marrow derived human MSCs [32], functional L-type I_{Ca} and TTX-sensitive I_{Na} were recorded in human and rat Sca-1⁺ MSCs, and in undifferentiated hESCs, but not in rabbit and mouse cells [15,17]. The diversity of results on functional expression of I_{Ca} , may stem not only from variation in voltage-clamp protocols employed, but also may arise from paracrine effects of two sets of cells upon each other in co-cultures of myocytes and undifferentiated stem cells. In this respect, significant enhancement in IGF-1 and VEGF concentrations was observed in co-culture medium of myocyte and CSCs, following 96 h in culture [7], and it was suggested that c-kit⁺ CSCs may have paracrine effects on cardiomyocytes leading to increased myocyte survival in co-cultured condition.

Consistent with this idea, increased Ca^{2+} current density in CPCeP was observed in both “conditioned” medium and co-culture of CPCeP and NRCM (Fig. 5), suggesting that the primary paracrine effect originates from NRCM affecting CPCeP. Since the pooled data showed considerable scatter, we considered 2 time intervals and quantified both the fraction of cells with significant I_{Ca} and the average I_{Ca} in these cells. This revealed that the average I_{Ca} of responsive cells consistently was larger after 4–7 days of culture than after 1–3 days (Fig. 5B). It also appeared that conditioned medium from NRCM-cultures was more effective than co-cultured NRCM in sustaining a high fraction of CPCeP with significant I_{Ca} . Possibly this may be related to the high density of mono-cultured NRCM that was used to produce conditioned medium or to limited survival of NRCM in the sparser co-cultures. Since the incidence of I_{Ca} in both mono- and co-cultures of CPCeP decreases in time, we speculate that sustained expression of I_{Ca} is acquired under paracrine influences only by a subset CPCeP. Irrespectively it was significant that after culturing 4–7 days, the average I_{Ca} in all the examined CPCeP was much larger with conditioned than with regular medium (1.49 ± 0.26 pA/pF, $n = 34$ vs. 0.40 ± 0.15 pA/pF, $n = 32$; $p < 0.001$). It is not as yet clear whether such paracrine effects become critical in growth, maturation, and survival of myocytes as they acquire the ability to develop functional gap junctions with surrounding CPCeP, or *vice-versa*. The importance of Pim-1 kinase was demonstrated in control experiments, which rarely revealed any significant I_{Ca} in CPC (Figs. 5B, C).

Thus we find that the expression of I_{Ca} is augmented both by expression of Pim-1 kinase and signals originating from NRCM.

4.3. Calcium signaling of co-cultured cells

Cardiac calcium signaling characteristics are an integral part of the functional cardiomyocyte phenotype. Both CPCeP and NRCM show rapid rises of Ca^{2+} of differing magnitudes following depolarizations produced by high KCl pulses (Fig. 7). The smaller

rise in intracellular Ca^{2+} in CPCeP was consistent with the electrophysiological findings of smaller expression of Ca^{2+} current density in CPCeP. Ca^{2+} -transients triggered by caffeine were observed in only a small fraction of CPCeP where they activated with some delay, and had slower rates of rise as compared to those measured in NRCM, suggestive of a slowly evolving calcium stores and CICR mechanism. In comparison, published results indicate that undifferentiated CSCs did not trigger significant caffeine-induced Ca^{2+} -transients and appeared not to express RyRs [18], but possessed robust expression of IP_3R and SERCA [29]. The finding of caffeine-induced Ca^{2+} -transients in only few CPCeP may reflect differing levels of cellular maturation in co-cultures or undetectable connections that may exist between CPCeP and NRCM allowing for transfer of Ca^{2+} waves from NRCM to CPCeP.

Both NRCM and CPCeP generated intracellular Ca^{2+} transients in response to applications of 100 μM of ATP. Although the rise of intracellular calcium occurred later in CPCeP, with time it reached higher levels than in NRCM. Similarly, robust IP_3 -mediated Ca^{2+} release mechanisms have been reported in human CPC [29], human mesenchymal system [33] and mouse embryonic stem cells [34,35]. Spontaneous elevations in the intracellular Ca^{2+} concentrations related to IP_3 -mediated Ca^{2+} release from SR were reported in human CPC, but no evidence for an association, between Ca^{2+} release from IP_3 stores and initiation of Ca^{2+} oscillations, was found in undifferentiated human CPC [29]. Application of ATP activated a Cl^- -dependent current in CPCeP, but the ability of ATP to activate intracellular rise in Ca^{2+} was independent of its membrane depolarizing effects since ATP continued to be effective in releasing calcium in zero calcium containing solutions (results not shown). IP_3 releases Ca^{2+} from SR in adult, embryonic and neonatal skinned or chemically permeabilized cardiomyocyte [13,36]. Coppi et al. also report an ATP-activated voltage-dependent inward and outward current in human MSCs. Since the current disappeared on application of K^+ -free solution, they suggested that the outward currents were carried by K^+ [37].

We also observed a Ni^{2+} -sensitive I_{NCX} current in undifferentiated CPCeP. Since expression of RyR2, SERCA and L-type Ca^{2+} currents increases with cardiomyocyte maturation while NCX levels remain stable [11], NCX activity is likely to contribute to the Ca^{2+} homeostasis in CSCs.

In summary, CPCeP appear to express channels and currents consistent with electrophysiological properties of cardiac calcium handling: functional I_{NCX} , L-type DHP-sensitive I_{Ca} and RyR2. These characteristics are critical for initiation of spontaneous calcium release activity, spontaneous beating and contractions, especially in pace maker cells of the heart. Maturation of calcium-handling properties is enhanced by co-cultures with NRCM or by conditioned medium of such co-cultures, indicative of paracrine effects of NRCM on CPCeP. Electrophysiological coupling between CPCeP and NRCM after co-culture strongly supports the expression of the gap junction protein, Cx43 between the two cell types. Thus, in the context of adoptive transfer to damaged myocardium, it is tempting to speculate that similar electromechanical contiguity is acquired by CPCeP when these cells are transplanted into an injured segment of myocardium, resulting in improvement of cardiac performance.

Acknowledgments

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Abbreviations

CICR	calcium-induced calcium release
C_m	membrane capacitance
CPC	cardiac progenitor cell
CPCeP	Cardiac progenitor cells engineered to express eGFP and Pim-1 kinase
CSC	cardiac stem cell
Cx43	connexin 43
DHPR	L-type calcium channels
eGFP	enhanced green fluorescent protein
ESC	embryonic stem cell
I_{ATP}	ATP-activated current
I_{Ca}	calcium current
IGF-1	insulin-like growth factor-1
I_{Na}	sodium current
I_{NCX}	sodium–calcium exchanger current
iPSC	induced pluripotent stem cell
NCX	sodium–calcium exchanger
NRCM	neonatal rat cardiomyocyte
RyR	ryanodine receptor
SERCA	sarco-endoplasmic reticulum calcium pump
SR	sarcoplasmic reticulum

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Fig. 1.

Configuration of CPCeP examined separately and in co-culture with NRCM. A–C: Images of single CPCeP cultured alone for 1–2 days (A) and 5–7 days (B) and with NRCM for 5 days (C). In panels A and B, the green fluorescence from eGFP is superimposed on the bright field images. D: Capacitive membrane currents of an isolated voltage-clamped CPCeP before (black) and after (red) decoupling of an adjacent NRCM. E: Average values of the total membrane capacitance measured in different voltage-clamp configurations. The icons, that are also used in the following figs., show a CPCeP at the end of a patch electrode, the same configuration with an attached cardiomyocyte (CPCeP + NRCM), and an isolated voltage-clamped cardiomyocyte (NRCM). F and G: Transfer of dye from a voltage-clamped

PCPeP (red and orange regions of interest) to an attached NRCM (blue and green regions of interest). F: Regions of interest (ROI) and sample frames measured at 4 and 14 min. G: Time course of normalized fluorescence intensity in the regions of interest.

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Fig. 2.

Prevalence membrane currents (I_{Ca} , I_{Na} , I_{ATP} , I_{NCX}) in voltage-clamped CPCeP cultured alone (panel D, red bars) or with NRCM (panel D, hatched bars). A: Co-cultured cells with eGFP-labeled CPCeP showing green fluorescence superimposed on the bright field image also showing associated NRCM and the voltage-clamp pipette (μ). B and C: Separately cultured CPCeP generated no detectable I_{Ca} when tested with a voltage-clamp protocol consisting of repeated step depolarizations of increasing amplitude (panel B), but generated a nifedipine-sensitive inward I_{Ca} (arrow) during brief ramp depolarizations (panel C). D: Prevalence of responsive cells. The numbers at the top of each bar (n_1/n_2) indicate the number of cells with clearly detectable current (n_1) out of the total number of tested cells (n_2). I_{Ca} in separately cultured CPCeP was probed using both step- (STEP) and ramp- (RAMP) protocols. I_{ATP} was measured at a fixed holding potential (HOLD), while I_{NCX} was recorded with a repolarizing ramp (RAMP).

Fig. 3. Calcium currents (I_{Ca}) in cardiac progenitor cell engineered to express eGFP and Pim-1 kinase (CPCeP, n = 8) attached to neonatal rat cardiomyocyte (NRCM) and in NRCM without (n = 6) or with (n = 3) attachments to CPCeP. A: Representative traces of I_{Ca} using voltage-step depolarizations to different test potentials from a holding potential of -40 mV. B: Average time-to-peak values of I_{Ca} . C: Current-voltage (I-V) relations for I_{Ca} normalized relative to the membrane capacitance. D: Average values of peak I_{Ca} measured at 0 mV in each group. Stars indicate significance levels (* $p < 0.05$, ** $p < 0.01$) while n indicates the number of voltage-clamped cells.

Fig. 4. Sodium currents (I_{Na}) in CPCeP attached to NRCM, in NRCM alone, and in NRCM attached to CPCeP. A: Sample traces of membrane current activated by step-depolarization from a holding potential of -80 mV. B: Average time-to-peak values of I_{Na} measured at the peak value of the I-V relations. C: I-V relations of the density of I_{Na} . D: Average peak values I_{Na} in each group.

Fig. 5. Paracrine effects of neonatal rat cardiomyocyte (NRCM) on I_{Ca} in cardiac progenitor cell engineered to express eGFP and Pim-1 kinase (CPCeP). A: Percentile distributions of the magnitudes of I_{Ca} in responsive CPCeP according to the conditions of the culturing process (regular medium vs. co-culture with NRCM vs. conditioned medium) and its duration (1–3 days or 4–7 days). B: Average I_{Ca} in responsive CPCeP (black symbols) and CPC (red symbols). C: Fraction of responsive CPCeP and CPC. The numbers next to each data indicate the number of responsive cells producing significant I_{Ca} (>0.4 pA/pF; B) and this number divided by the total number of voltage-clamped cells (C).

Fig. 6. $\text{Na}^+-\text{Ca}^{2+}$ exchanger currents (I_{NCX}) in neonatal rat cardiomyocyte (NRCM, $n = 6$), cardiac progenitor cell engineered to express eGFP and Pim-1 kinase (CPCeP) cultured alone ($n = 9$) or attached ($n = 7$) to NRCM. A and B: Representative current traces (A) and I-V relations (B) measured with ramp-clamp protocol (inset) in the absence (black) and presence of 5 mM Ni^{2+} (red). C: Bar graph showing the densities of the Ni^{2+} -sensitive I_{NCX} at +80 and -120 mV.

Fig. 7.

Calcium transients in co-cultured CPCeP and NRCM. Panels A–C show details of the Ca^{2+} transients elicited by 10 mM caffeine (A), 100 mM KCl (B) and 100 μM ATP (C). The graphs on the left show the time course of the Ca^{2+} signals (F/F_0) measured in color-coded regions of interest (panel E) corresponding to NRCM (red and orange) and CPCeP (light blue, dark blue, green, olive, purple, violet). The sample frames in panels A–C show ratiometric images recorded during (1) and after the interventions (2) at the times indicated in the graphs. D: Baseline fluorescence (F_0). F: Average Ca^{2+} transients (F/F_0-1) evoked by caffeine (Caff), KCl-depolarization (KCl) and ATP (ATP) in multiple NRCM (red bars) and CPCeP (blue bars). The cells were imaged confocally at a frame rate of 30 Hz after incubation with Fluo-4 AM.

Fig. 8. ATP-activated currents (I_{ATP}) in CPCeP (n = 9), CPCeP attached to NRCM (n = 13), and NRCM alone (n = 9). A: Representative traces of I_{ATP} in individual CPCeP and NRCM. B: Averages of maximum I_{ATP} current density in each group. Cells were voltage-clamped to -80 mV for 8 s and exposed to 100 μ M ATP for 1 s.

Fig. 9.

Immunostaining of connexin 43 (Cx43, red) in eGFP-expressing CPCeP (green) cultured alone (A–D) or together with NRCM (E–G). Details of panels A and E are shown in panels B and F, respectively, where the red color corresponding to Cx43 staining is accentuated by being shown within the outlines of the CPCeP (dashed white lines) without the green color that identified these cells. Clustering of Cx43 (arrows) on the boundaries between cells is illustrated in panels C (CPCeP–CPCeP), D (CPCeP–*) and G (CPCeP–NRCM; arrows). Scale bars in white are 10 μm.