Chapter 4

Investigation of the Pro-arrhythmic Features of Pathological Cardiac Hypertrophy in Neonatal Rat Ventricular Cardiomyocyte Cultures

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Abstract

Background: Different factors may trigger arrhythmias in diseased hearts including fibrosis, cardiomyocyte hypertrophy, hypoxia and inflammation. This makes it difficult to establish the relative contribution of each of them to the occurrence of arrhythmias. Accordingly, in this study, we used an *in vitro* model of pathological cardiac hypertrophy (PCH) to investigate its pro-arrhythmic features and the underlying mechanisms independent of fibrosis or other PCH-related processes.

Methods and Results: Neonatal rat ventricular cardiomyocyte (nr-vCMC) monolayers were treated with phorbol 12-myristate 13-acetate (PMA) to create an *in vitro* model of PCH. Electrophysiological properties of PMA-treated and control monolayers were analyzed by optical mapping at day 9 of culture. PMA treatment led to a significant increase in cell size and total protein content. It also caused a reduction in sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 level (32%) and an increase in natriuretic peptide A (42%) and α1-skeletal muscle actin (34%) levels indicating that the hypertrophic response induced by PMA was indeed pathological in nature. PMA-treated monolayers showed increases in action potential duration (APD) and APD dispersion, and a decrease in conduction velocity (CV; APD_{30} of 306±39 vs 148±18 ms, APD_{30} dispersion of 85±19 vs 22±7 and CV of 10±4 vs 21±2 cm/s in controls). Upon local 1-Hz stimulation, 53.6% of the PMA-treated cultures showed focal tachyarrhythmias based on triggered activity (n=82), while control group showed 4.3% tachyarrhythmias (n=70).

Conclusion: Following PMA treatment, nr-vCMC cultures acquire features of pathologically hypertrophied hearts and show a high incidence of focal tachyarrhythmias associated with APD prolongation and an increase in APD dispersion. PMA-treated nr-vCMC cultures may thus represent a well-controllable *in vitro* model for testing new therapeutic interventions targeting specific aspects of hypertrophy-associated arrhythmias.

Key words: phorbol 12-myristate 13-acetate, pathological cardiac hypertrophy, triggered activity, cell culture

Introduction

An increase in cardiac demand triggers the heart to respond in several ways, including by the enlargement of cardiomyocytes. Such cardiac hypertrophy is essentially a beneficial compensatory process as it decreases wall stress, while increasing cardiac output.[1] This adaption by growth occurs under physiological conditions like exercise and pregnancy, but also in response to myocardial infarction and other cardiac pathologies. Whereas physiological cardiac hypertrophy is usually reversible and contributes to optimal heart function, hypertrophy due to cardiac disease (*i.e.* pathological cardiac hypertrophy [PCH]) is typically associated with several irreversible time-dependent detrimental changes, including maladaptive remodeling of cardiac structure, metabolism, electrophysiology and ion homeostasis, which may ultimately culminate in heart failure.[2,3] Electrophysiological remodeling, especially if sustained, imposes an increased risk of developing cardiac arrhythmias. The relationship of PCH with ventricular tachyarrhythmias has been investigated in whole heart mapping studies.[4-7] However, the complexity of three-dimensional (3D) myocardial tissue and the presence in pathologically hypertrophied hearts of various other changes in cardiac structure and function including fibrosis, inflammation and metabolic remodeling,[7,8] complicates assessment of the specific contribution of PCH to the development of heart rhythm disturbances. This problem can be overcome by using two-dimensional (2D) cell culture models of defined composition to study PCH-related pro-arrhythmic changes. Induction of hypertrophy-related pathological changes in cardiomyocyte cultures can be accomplished by exposure of the cells to a variety of different peptide and non-peptide hormones and growth factors including angiotensin II (AngII), endothelin 1 (ET-1) and certain natural and synthetic catecholamines.[9] Many of these molecules exert their pro-hypertrophic effects through the activation of phospholipase C leading to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 to specific receptors located in the membranes of the sarcoplasmic reticulum and in the nuclear envelope causes Ca^{2+} release into the cytosol and nucleus and activation of several pro-hypertrophic factors including calcineurin, nuclear factor of activated T cells (NFAT) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).[10] DAG, on the other hand, stimulates cardiac hypertrophy mainly by acting as stimulatory cofactor of protein kinase C (PKC) and protein kinase D (PKD).[11,12] The pro-hypertrophic effects of DAG can be mimicked by phorbol 12-myristate 13-acetate (PMA). Indeed,

treatment of neonatal rat ventricular cardiomyocytes (nr-vCMCs) with PMA has been shown to induce a gene expression program in these cells sharing many features with that of pathologically hypertrophied hearts.[13-15] Prominent among the PMA-induced changes are those involving the expression level, cellular localization and specific activity of ion channels and transporters,[16,17] connexins,[18,19] and Ca^{2+}-handling proteins[20-22] similar to what happens in pathologically hypertrophied hearts. Still, relatively little is known about the possible pro-arrhythmic consequences of these changes, which are commonly referred to as electrical remodeling. Therefore, the purpose of the present study was to investigate in PMA-treated nr-vCMC cultures the contribution of PCH to the development of cardiac arrhythmias independent of fibrosis, inflammation and hypoxia.

Materials and Methods

All animal experiments had the approval of the Animal Experiments Committee of the Leiden University Medical Center and complied with the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

Cardiomyocyte isolation

nr-vCMCs were isolated and cultured essentially as described previously.[23] In brief, 2-day-old Wistar rats were anaesthetized with 4–5% isoflurane. After confirmation of anesthesia, the chest was opened, the heart was excised and the ventricles were separated from the remainder of the cardiac tissue. Next, the ventricular myocardium was cut into small pieces (~1 mm) and further dissociated by incubation at 37°C with a buffer solution containing 0.01 mM $CaCl_2$, 5 mM $MgCl_2$, 450 units/ml of collagenase 1 (Worthington, Lakewood, NJ, USA) and 18.75 Kunitz units/ml DNAse I (Sigma-Aldrich, St. Louis, MO, USA). Cells and remaining tissue fragments were pelleted by centrifugation at 161 × *g* and room temperature (RT) for 10 min and resuspended in growth medium (Ham's F10 medium supplemented with 10% fetal bovine serum [FBS] and 10% horse serum [HS; all from Life Technologies, Bleiswijk, the Netherlands]). The cell suspension was then applied to Primaria culture dishes (Corning Life Sciences, Amsterdam, the Netherlands) and incubated for 75 min in a humidified incubator at 37ºC and 5% CO_2 to allow preferential attachment of non-

myocytes. The unattached cells (mainly cardiomyocytes) were collected, passed through a cell strainer (70-µm mesh pore size; BD Biosciences, Breda, the Netherlands) to obtain a single cell suspension and applied at a density of 6×10^5 cells/well of a 24-well cell culture plate (Corning Life Sciences) to fibronectin (Sigma-Aldrich)-coated, round glass coverslips (15-mm diameter). One day later (*i.e.* at culture day 1), the cells were treated in growth medium with mitomycin C (10 µg/ml; Sigma-Aldrich) for 2 h to inhibit proliferation of remaining non-myocytes. The growth medium was subsequently replaced by a 1:1 mixture of Dulbecco's modified Eagle's medium [DMEM; Life Technologies] and Ham's F10 medium supplemented with 5% HS, 2% bovine serum albumin (BSA) and sodium ascorbate to a final concentration of 0.4 mM. This so-called maintenance medium was refreshed daily. To induce pathological hypertrophy cultures were exposed to 1 µM PMA (BioVision, Milpitas, CA, USA) for 24 h at day 3 and 8 of culture.

Optical mapping

Optical mapping was done at day 9 of culture. Prior to optical mapping, the cells were incubated for 10 min in maintenance medium containing 8 µM of the voltage-sensitive dye di-4-ANEPPS (Life Technologies) and given fresh medium consisting of DMEM/HAM's F12 (Life Technologies) without phenol red and serum. Immediately afterwards, cultures were optically mapped at 37°C using a MiCAM ULTIMA-L imaging system (SciMedia, Costa Mesa, CA, USA). To allow for a fair comparison of action potential duration (APD) and conduction velocity (CV), all cultures were locally stimulated at 1 Hz using an epoxy-coated bipolar platinum electrode with square suprathreshold (*i.e.* 8 V) electrical stimuli of 10 ms. Parameters of interest were calculated using Brain Vision Analyzer 1208 software (Brainvision, Tokyo, Japan). Optical signals of 9 pixels were averaged to minimize noise artifacts. To calculate CV, two 3 by 3 pixel grids located 2-8 mm apart on a line perpendicular to the activation wavefront were used. APD was calculated at 30% (APD_{30}) and 80% (APD_{80}) of repolarization. CV and APD values were averages of values obtained from 6 different positions equally distributed across the cell cultures. APD_{30} and APD_{80} dispersion were expressed as the standard deviation (SD) of the mean of the APDs. For determining CV, APD and APD dispersion only nr-vCMC cultures with uniform activation patterns were used. Occurrence of pro-arrhythmic features was

also evaluated after 1-Hz local stimulation. An early afterdepolarization (EAD) was defined as a reversal of repolarization during phase 2 or 3 of the AP of more than 10% of the maximum optical signal amplitude. A focal tachyarrhythmia was defined as an activation pattern in which an EAD was followed by 2 or more uninterrupted oscillations in membrane potential without giving rise to a reentrant circuit.

Immunocytology

For immunostaining, 8×10^4 cells were seeded on fibronectin-coated, round glass coverslips (15-mm diameter) in wells of 12-well cell culture plates (Corning Life Sciences). At day 9 of culture, cells were fixed by incubation for 30 min in phosphate-buffered 4% formaldehyde (Klinipath, Duiven, the Netherlands) and permeabilized by a 20-min treatment with 0.1% Triton X-100 in phosphate-buffered saline (PBS) both at RT. Next, cells were incubated overnight at 4°C with mouse anti-sarcomeric α-actinin (clone: EA-53; Sigma-Aldrich), mouse anti-sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 (Serca2; clone: 2A7-A1; Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-natriuretic peptide precursor type A (Nppa; Merck Millipore, order number: AB5490) and rabbit anti-α1-skeletal muscle actin (Acta1; Abcam, Cambridge, United Kingdom, order number: ab52218) primary antibodies diluted 1:200 in PBS + 0.1% donkey serum (Santa Cruz Biotechnology, Dallas, TX, USA) followed by a 2-h incubation at RT with appropriate Alexa Fluor 488- or 568-conjugated donkey IgG (H+L) secondary antibodies (Life Technologies) diluted 1:400 in PBS + 0.1% donkey serum. Nuclei were counterstained with 10 µg/ml Hoechst 33342 (Life Technologies) in PBS. Cells were washed three times with PBS after fixation, permeabilization and incubation with primary antibody, secondary antibody and DNA-binding fluorochrome. To minimize photobleaching, coverslips were mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Pictures were taken with a fluorescence microscope equipped with a digital color camera (Nikon Eclipse 80i; Nikon Instruments Europe, Amstelveen, the Netherlands) using NIS Elements software (Nikon Instruments Europe). Cell surface area (CSA) and fluorescent intensity were measured using dedicated software (ImageJ, version 4.1 National Institutes of Health, USA).

Statistical analysis

Different experimental groups were compared using the unpaired samples *t*-test. Data represented as mean±SD. Differences among means were considered significant at $P \leq 0.05$. Graphs were prepared in GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA).

Results

PMA induces a hypertrophic response in nr-vCMCs

Immunostaining of control and PMA-treated nr-vCMC cultures for sarcomeric α-actinin showed the presence of ~13% non-myocytes in the cultures (Figure 1A). PMA treatment of nr-vCMC cultures increased mean CSA by 30% (3.5±1.5 [n=81] vs 2.6±1.0 pixels in control cultures [n=54], $P<0.0005$) (Figure 1B) and total protein content by 80% (1.3±0.1 vs 0.7±0.1 mg/10^7 cells in control cultures [n=9 for both experimental groups], $P<0.0001$) (Figure 1C), confirming that PMA is a hypertrophy inducer.

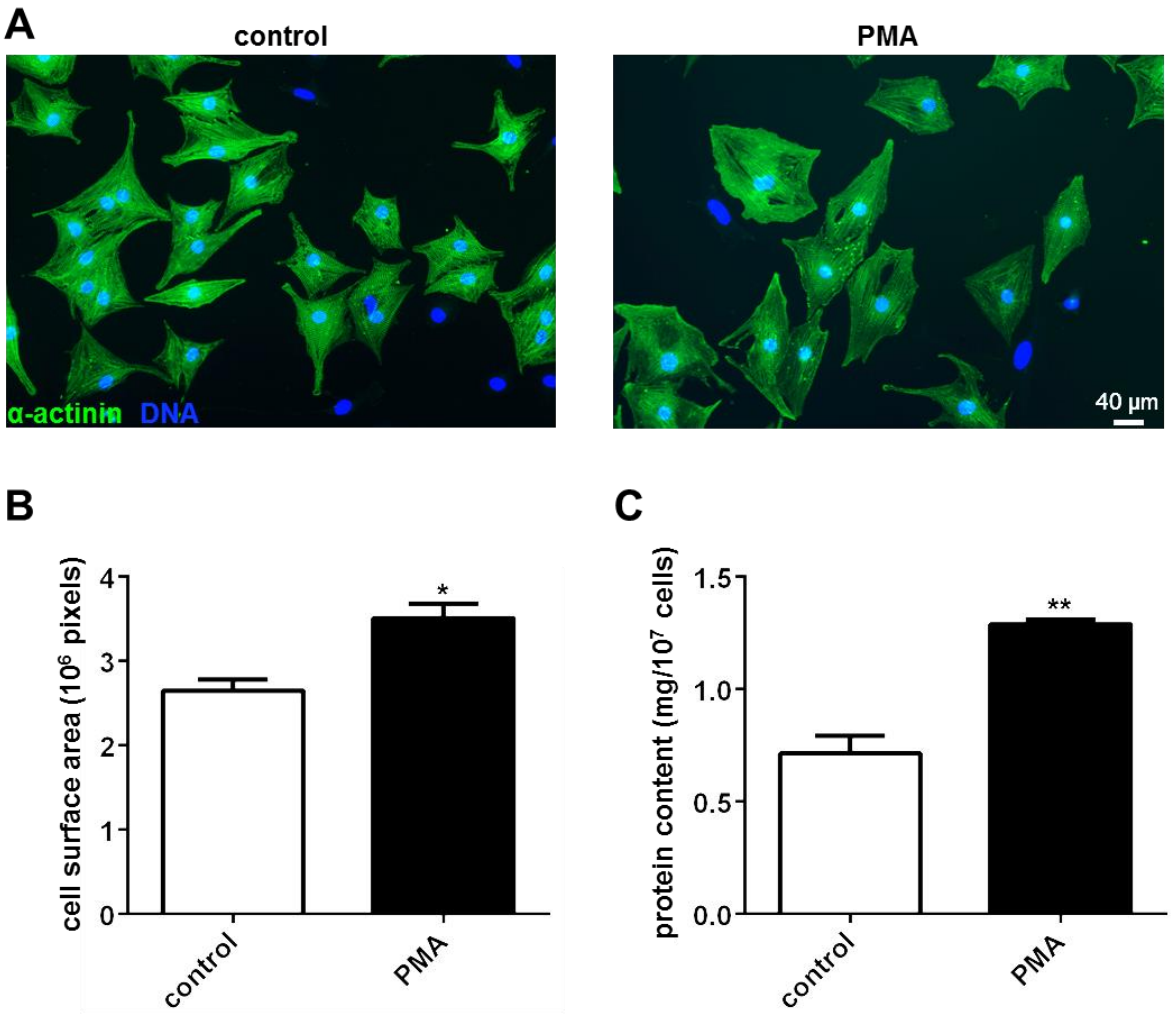

Figure 1. Characterization of PMA-treated nr-vCMC cultures. (**A**) α-actinin staining showing the presence of 13% non-myocytes in the nr-vCMCs cultures. Quantification of (**B**) CSA and (**C**) protein content of control and PMA-treated nr-vCMCs. CSA and protein level were increased in cultures treated with PMA indicative of a hypertrophic response. * P<0.0005 and ** P<0.0001.

PMA-treated nr-vCMCs acquire a pathological hypertrophy-related phenotype

To investigate the nature of the hypertrophic response induced by PMA, control and drug-treated nr-vCMC cultures were immunostained for markers distinguishing physiological from pathological hypertrophy. The PMA-treated nr-vCMC cultures displayed a 42% increase in Nppa level (17.8±2.0 vs 12.5±4.4 arbitrary units, P<0.005) (Figure 2A), a 34% increase in Acta1 expression (22.0±4.1 vs 16.4±2.6 arbitrary units, P<0.0005) (Figure 2B) and a 32% decrease in Serca2a level (15.9±2.5 vs 21.1±3.5 arbitrary units, P<0.0001) (Figure 2C) as compared to those in

control cultures. This indicates that PMA-treated nr-vCMCs obtain properties of pathologically hypertrophic cardiac muscle cells.

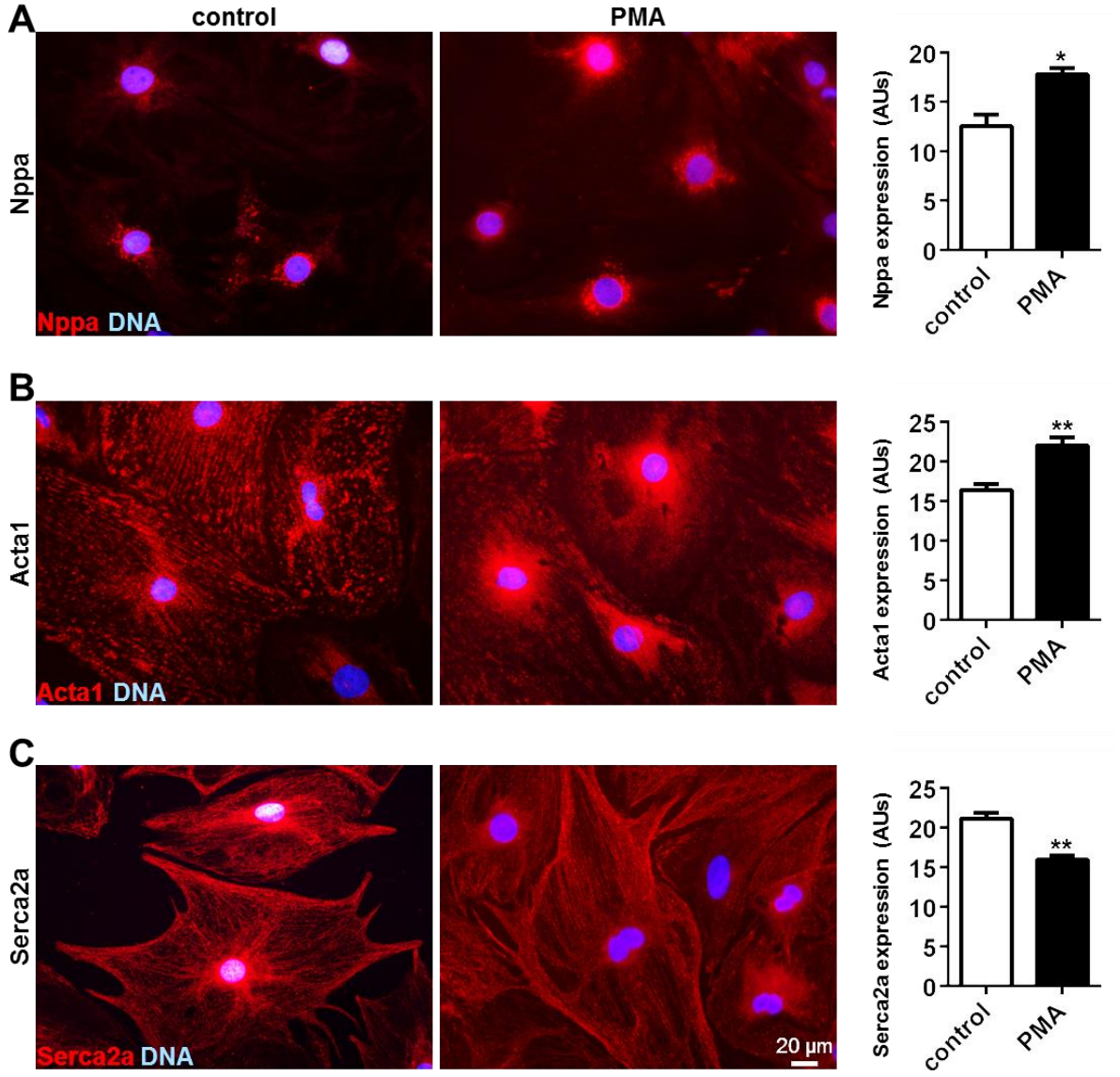

Figure 2. PMA treatment endows nr-vCMCs with features of pathological hypertrophied ventricular myocardium including an increase in (**A**) Nppa (42%) and (**B**) Acta1 levels (34%) and a decrease in (**C**) Serca2a expression (32%). * $P<0.005$ and ** $P<0.0005$. AUs, arbitrary units.

Conduction and repolarization are slowed by PMA treatment of nr-vCMCs cultures

Optical mapping recordings in 1 Hz-paced, uniformly propagating nr-vCMC cultures showed that PMA treatment causes a strong reduction in CV (10±4 vs 21±2 cm/s in control cultures, $P<0.0001$) (Figure 3A). Moreover, the PMA-treated nr-vCMC

cultures displayed a large increase in APD_{30} (306±39 vs 148±18 ms in control cultures, $P<0.0001$) and APD_{80} (516±53 vs 225±34 ms in control cultures, $P<0.0001$) (Figure 3B,C and Figure 4A). Spatial APD dispersion was also increased in PMA-treated cultures (APD_{30} dispersion of 85±19 vs 22±7 ms in control cultures, $P<0.0001$ and APD_{80} dispersion of 50±9 vs 25±2 ms in control cultures, $P<0.0001$) implying increased heterogeneity of repolarization (Figure 3B,C).

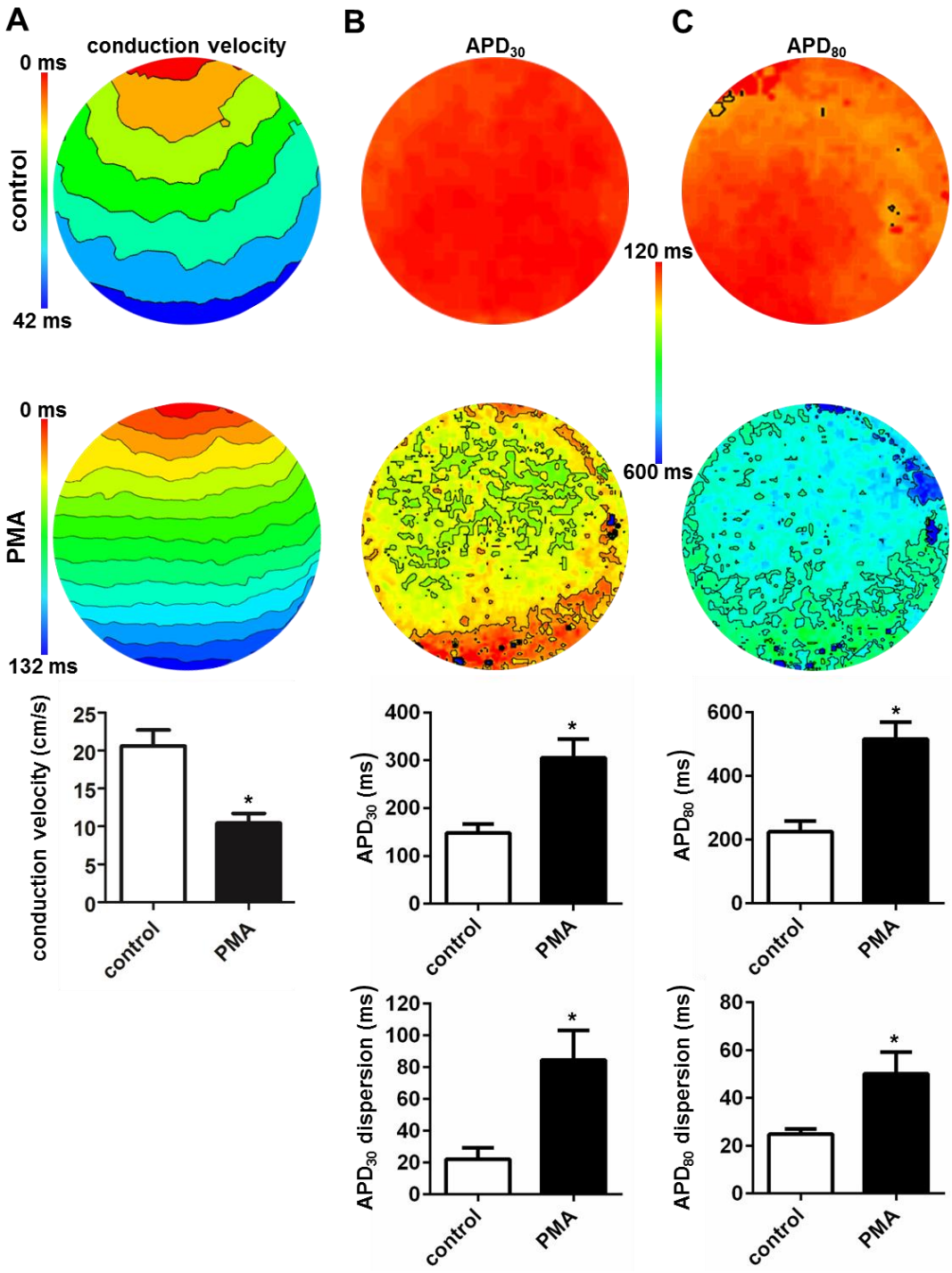

Figure 3. PMA treatment of nr-vCMC cultures causes conduction slowing and heterogeneous APD prolongation. (**A**) Typical activation maps with 6-ms isochronal spacing and corresponding quantitative assessment of control and PMA-treated nr-vCMC cultures showing slowing of conduction upon PMA treatment. Typical APD_{30} (**B**) and APD_{80} (**C**) map of control and PMA-treated nr-vCMC cultures and corresponding quantitative assessments of APD and APD dispersion showing PMA-induced increases in APD and APD dispersion. Cultures were subjected to electrical point stimulation at a frequency of 1-Hz. * P<0.0001.

Focal triggered activity is a prominent pro-arrhythmic feature of PMA-treated nr-vCMC cultures

The PMA-induced APD prolongation and increase in APD dispersion provided enough time and depolarizing force, respectively, for formation of EADs in the drug-treated cultures, which could oscillate repetitively resulting in focal tachyarrhythmias. The incidence of this type of arrhythmias following local 1-Hz stimulation was 53.6% in the PMA-treated nr-vCMC cultures (n=82) while control nr-vCMC cultures showed 4.3% arrhythmias (n=70) (Figure 4A, B). During focal tachyarrhythmias, repolarization halted at the initiation site of the EAD (Figure 4C, point 1) followed by slow repolarization in areas in the vicinity of the region of sustained depolarization, which favored EAD formation (Figure 4,C point 2).

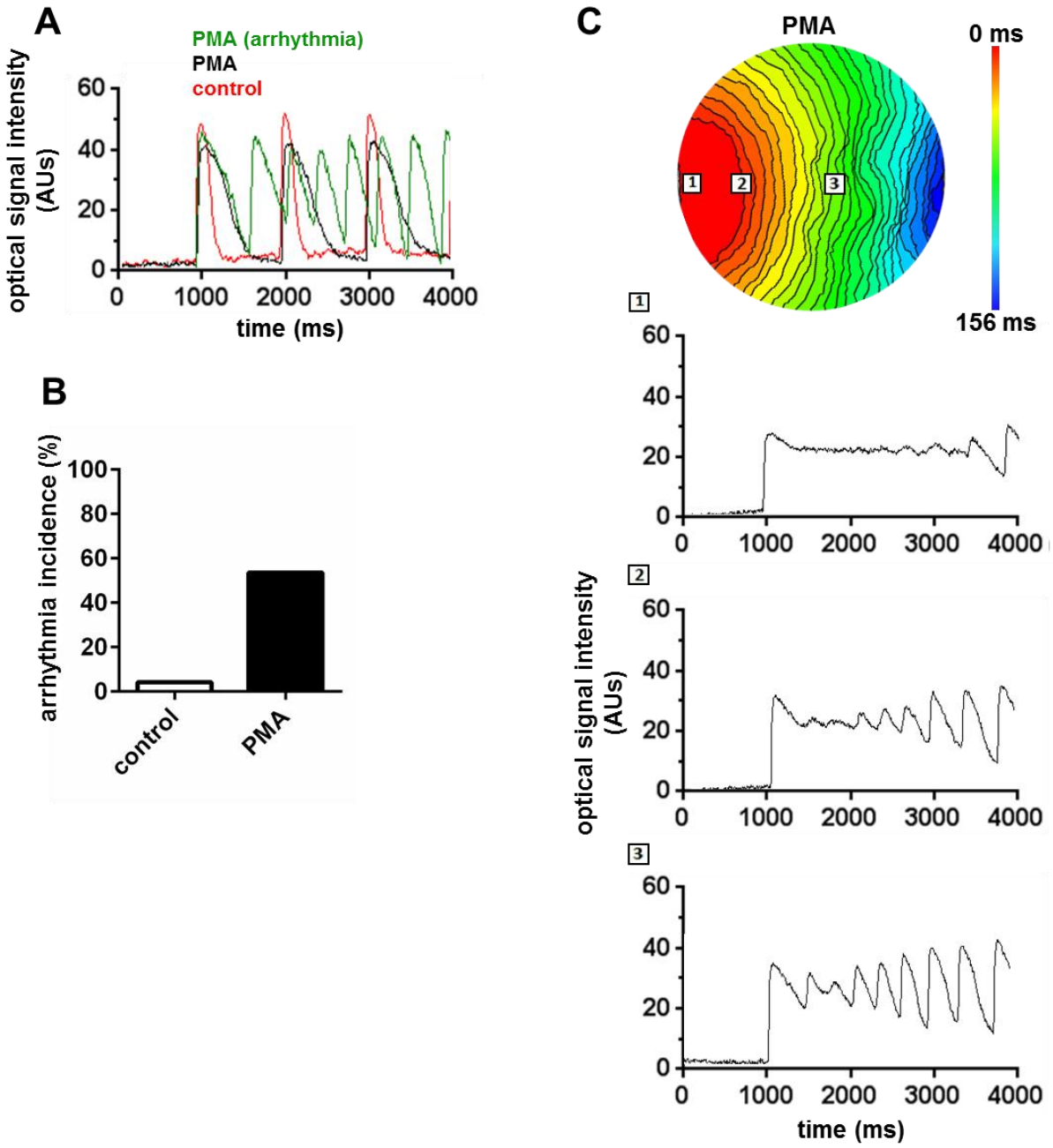

Figure 4. PMA-treated nr-vCMC cultures show a high incidence of focal tachyarrhythmias. (**A**) Typical optical signals from control (red) and PMA-treated cultures showing APD prolongation (black) and onset of tachyarrhythmia (green). (**B**) Quantification of arrhythmia incidence (*i.e.* incidence of focal tachyarrhythmias) in control and PMA-treated nr-vCMC cultures. (**C**) Activation map of a PMA-treated nr-vCMC culture displaying triggered activity. Corresponding optical signals showing ceased repolarization (point 1), EAD initiation (point 2) and EAD propagation (point 3). AUs, arbitrary units.

Discussion

The major findings of this study are: (i) Exposure of nr-vCMC cultures for 2 times 24 hours to PMA induces a hypertrophic response in the cardiac myocytes with hallmarks of pathological hypertrophied ventricular myocardium; (ii) Following prolonged PMA treatment nr-vCMC cultures undergo electrical remodeling as evinced by a decrease in CV and an increase in APD and APD dispersion; (iii) PMA-treated nr-vCMC cultures display a high incidence of triggered activity causing focal tachyarrhythmias; (iv) Mechanistically, the arrhythmias observed in nr-vCMC cultures rendered pathologically hypertrophic by prolonged PMA treatment are probably a direct consequence of the electrical remodeling process.

In vitro models of PCH

In recent years much has been learned about the signaling pathways orchestrating both physiological and pathological heart growth.[24] Through extensive molecular, genetic and pharmacological studies, G protein-coupled receptors (GPCRs) and their ligands (*e.g.* AngII, ET-1, noradrenaline [NE]) have been identified as key regulators of PCH.[25] This has led researchers to use these ligands or synthetic analogs hereof (*e.g.* isoproterenol, phenylephrine [PE]) to develop *in vitro* and *in vivo* models of PCH and heart failure.[9,26-28] In the *in vitro* models of pathological ventricular hypertrophy, an increase in CSA, mRNA and protein content, cell capacitance and/or protein synthesis rate was taken as proof of cell growth while increases in the expression of Nppa, natriuretic peptide precursor B (Nppb), Acta1, β-myosin heavy chain (Myh7) and/or decreases in α-myosin heavy chain (Myh6) and Serca2a levels were considered indicative of a pathological rather than a physiological hypertrophic response.

The pro-hypertrophic effects of AngII-, ET-1- and cathecholamine-binding GPCRs in the heart are for a large part attributable to the activation of phospholipase C, which converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers IP_3 and DAG. In this study, PCH was induced by using PMA as a synthetic analogue of the second messenger DAG instead of by agonist-induced GPCR activation. Possible pro-hypertrophic effects caused by direct ligand-induced

IP_3 receptor (IP_3R) activation and subsequent Ca^{2+} release are therefore expected to be absent in our nr-vCMC-based PCH model. The PMA-treated nr-vCMC cultures nevertheless show a very robust hypertrophic response with hallmarks of PCH (Figures 1 and 2), which is consistent with the results of previous studies that employed (prolonged) PMA treatment to render cardiomyocyte cultures hypertrophic.[17,20,29,30] This suggests that either IP_3R signaling is not necessary for inducing PCH-related phenotypic changes in cultured nr-vCMCs or that IP_3Rs get activated by DAG-dependent signaling. Indeed, several studies have identified the DAG receptors PKC and PKD as important mediators of (pathological) cardiac hypertrophy and fetal cardiac gene reactivation.[14,21,31-34] Consequently, overexpression of PKCα[35] or PKD3[36] in nr-vCMCs induced pathological hypertrophic growth with increased fetal cardiac gene expression in these cells.

Pro-arrhythmic mechanisms of PCH

Despite the rapidly increasing knowledge about the molecular pathways involved in the development of PCH and heart failure, the mechanisms underlying electrical remodeling of the diseased heart are still poorly understood. This is partially due to the disparate results obtained in different *in vivo* studies focusing on PCH-related changes in cardiac electrophysiology.[37-39] A confounding factor in these studies has been the use of different experimental conditions, animal models and/or patient groups with distinct contributions of other factors besides PCH to the electrophysiological remodeling process. In this study, using a well-defined *in vitro* model system, heterogeneous APD prolongation and EAD-triggered activity were identified as likely key players in the development of PCH-associated arrhythmias. These findings are consistent with the results of animal and clinical studies attributing a prominent role of EADs to the development of ventricular tachyarrhythmias.[40-43] In our study, we did not investigate the ionic basis of the EADs. However, given the pronounced elongation of phase 2 of the AP in the PMA-treated nr-vCMCs, L-type Ca^{2+} channels and delayed rectifier K^+ channels[43] are probably involved. In support of this idea, Puglisi *et al.* recently showed that chronic exposure (*i.e.* for 48-72 hours) of nr-vCMCs to PMA caused a strong decrease in normalized $I_{Ks.}$[17] The same researchers also reported a significant decrease in I_{to} and a substantial increase in $I_{Na+/Ca2+}$ while the normalized $I_{Ca,L}$ did not change

significantly. A decrease in I_{to} following overnight exposure of nr-vCMCs to PMA has also been documented by Walsh *et al.*[44] Another important result was the PMA-induced 32% decrease in Serca2a protein level. Similar findings were made by Porter *et al.* and Qi *et al.* following prolonged treatment of nr-vCMCs with PMA.[20,21] In their study, Qi and co-workers found that the reduction in Serca2a expression led to a slowing of diastolic Ca^{2+} uptake into the sarcoplasmic reticulum with possible pro-arrhythmic consequences. Besides the reduced repolarization reserve at the earlier phases of repolarization (*i.e.* between -40 and 0 mV) and the increased APD dispersion, also the conduction slowing observed in our PMA-treated nr-vCMC cultures is a major risk factor for the development of ventricular tachyarrhythmias.[45] The most likely explanation for this conduction slowing is gap junctional remodeling in combination with a downregulation of connexin 43 protein level as has been observed in patients with PCH or heart failure.[46,47]

PCH versus heart failure model

In response to hemodynamic stress and/or myocardial injury (*i.e.* when cardiac load exceeds cardiac output) the heart engages in a process called compensatory hypertrophy through the enlargement of cardiomyocytes by the parallel (concentric hypertrophy) or serial (eccentric hypertrophy) addition of sarcomeres. This process is under neurohormonal control of the adrenergic nervous system and renin-angiotensin system. At the molecular level, the changes in cardiomyocyte phenotype are accompanied by reinduction of the so-called fetal gene program, because patterns of gene expression mimic those observed during cardiac development. In the continued presence of pathologically stimuli, excessive cardiomyocyte death will provoke transition to dilated cardiomyopathy leading to heart failure. The latter process is associated with functional perturbations of cellular Ca^{2+} homeostasis and ionic currents resulting in impaired force generation and the development of malignant arrhythmias.[38,48] The PMA-treated nr-vCMC cultures display many of the same electrophysiological changes found in failing ventricular myocardium including a reduction in CV, heterogeneous APD prolongation and a high incidence of triggered electrical impulses.[3] This may suggest that the PMA-treated nr-vCMC cultures represent a relatively late stage in the transition from PCH to heart failure. Further evidence for this presupposition should come from a comparison of the

contractile force-generating capacity of control and PMA-treated nr-vCMC cultures and from comparative transcriptome analyses.

Conclusion

In the present study, treatment of nr-vCMCs for 2 times 24 hours with PMA not only promoted cardiomyocyte hypertrophy but also led to the reactivation of fetal cardiac genes as evinced by PMA-dependent increases in Nppa and Acta1 levels and a decrease in Serca2a expression. PMA-treated nr-vCMCs showed a high incidence of triggered tachyarrhythmias associated with increases in APD and APD dispersion caused by electrical remodeling. To the best of our knowledge, this is the first study in which the pro-arrhythmic features of PCH per se have been investigated. Since this *in vitro* model of PCH is highly controllable and provides reproducible results, it is ideally suited for testing, in proof-of-concept studies, new therapeutic interventions (genetic modifications or pharmacological treatments) targeting specific aspects of hypertrophy/heart failure-associated arrhythmias.

Study limitations

In this study, PCH-related arrhythmias were investigated in 2D cultures of nr-vCMCs. Although this *in vitro* model system has greatly contributed to our current understanding of heart structure and function and lends itself very well to pharmacological and genetic manipulation, the electrophysiological properties of PMA-treated nr-vCMCs will only partially resemble those of cardiomyocytes in the pathologically hypertrophied human heart. Also, in isolation, pathological hypertrophy may have a different impact on cardiomyocytes' behaviour than in combination with other cardiac pathologies like inflammation, hypoxia and fibrosis. Accordingly, discoveries made in PMA-treated nr-vCMC cultures will always have to be verified in more clinically relevant settings. Even so, due to the relative ease with which nr-vCMCs can be obtained, cultured and manipulated they represent a highly useful model system for mechanistic and therapy-directed cardiac research.

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Author contributions

Conceived and designed the experiments: ZN, BOB, DAP, AAF. Performed the experiments: ZN. Analyzed the data: ZN, BOB, DAP, AAF. Contributed reagents/materials/analysis tools: BOB, MJS, DAP, AAF. Wrote the paper: ZN, MJS, DAP, AAF.

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