Optogenetic activation of Gq signalling modulates pacemaker activity of cardiomyocytes

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Received 15 July 2013; revised 11 February 2014; accepted 13 February 2014; online publish-ahead-of-print 27 February 2014

Time for primary review: 28 days

Aims

Investigation of Gq signalling with pharmacological agonists of Gq-coupled receptors lacks spatio-temporal precision. The aim of this study was to establish melanopsin, a light-sensitive Gq-coupled receptor, as a new tool for the investigation of spatial and temporal effects of Gq stimulation on pacemaking in cardiomyocytes at an early developmental stage.

Methods and results

A vector for ubiquitous expression of melanopsin was tested in HEK293FT cells, which showed light-induced production of inositol-1,4,5-trisphosphate and elevation of intracellular Ca2+ concentration. Mouse embryonic stem cells were stably transfected with this plasmid and differentiated into spontaneously beating embryoid bodies (EBs). Cardiomyocytes within EBs showed melanopsin expression and illumination (60 s, 308.5 nW/mm2, 470 nm) of EBs increased beating rate within 10.2 ± 1.7 s to 317.1 ± 16.3% of baseline frequency. Illumination as short as 5 s was sufficient for generating the maximal frequency response. After termination of illumination, baseline frequency was reached with a decay constant of 27.1 ± 2.5 s. The light-induced acceleration of beating frequency showed a sigmoidal dependence on light intensity with a half maximal effective light intensity of 41.7 nW/mm2. Interestingly, EBs showed a high rate of irregular contractions after termination of high-intensity illumination. Local Gq activation by illumination of a small region in a functional syncytium of cardiomyocytes led to pacemaker activity within the illuminated area.

Conclusions

Light-induced Gq activation in melanopsin-expressing cardiomyocytes increases beating rate and generates local pacemaker activity. We propose that melanopsin is a powerful optogenetic tool for the investigation of spatial and temporal aspects of Gq signalling in cardiovascular research.

Keywords

Optogenetics • Cardiomyocyte • G proteins • Pacemaker

1. Introduction

The Gq-signalling pathway integrates diverse extracellular stimuli into intracellular responses. A huge variety of neurotransmitters and hormones activate Gq protein-coupled receptors leading to stimulation of phospholipase C-β (PLC-β) via the Gα subunit of the heterotrimeric Gq protein. PLC-β catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate producing the second messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 binds to IP3 receptors in the endoplasmatic or sarcoplasmatic reticulum and thereby initiates the release of Ca2+ from intracellular stores. Diacylglycerol leads to activation of protein kinase C, but can also activate canonical transient receptor potential channels, which lead to Ca2+ entry into the cell (Figure 1A).1–3

The Gq-signalling cascade plays an important role in diverse physiological and pathological conditions of the heart.4,5 In the developing cardiomyocytes, IP3 mediates cyclic intracellular Ca2+ release that is involved in the initiation and regulation of spontaneous electrical activity.6–10 IP3 receptors are also expressed in the adult sinus node of mice and application of Gq-coupled agonists, or IP3 causes an increase offiring rate indicating the contribution of the Gq-signalling cascade to pacemaking of the adult heart.11,12 Furthermore, Gq signalling seems to play a role in the generation of arrhythmias in atrial and ventricular cardiomyocytes.5,13,14 In addition to these acute effects, sustained activation of Gq protein-coupled receptors by endothelin-1 (ET-1) or phenylephrine has been shown to cause cardiomyocyte hypertrophy.5,15

For the investigation of Gq signalling, it is necessary to identify Gq-coupled receptors in the cell type of interest and to activate these by application of specific agonists. However, with the currently available methods, the analysis of temporal and spatial aspects of Gq signalling is technically limited because of non-localized and delayed action of agonists in vitro as well as in vivo. We propose that optogenetic Gq activation...
is a novel tool to investigate Gq signalling in the cardiovascular system with high temporal and spatial resolution.

Optogenetics is a technology that combines the expression of light-sensitive proteins with stimulation of cells and organs by light, and can be used in vitro as well as in vivo. Optogenetic stimulation allows a very high spatio-temporal resolution because light can be focused to specific regions using lenses or light guides, and the exact time of activation and deactivation is defined by onset and duration of illumination. By expression of optogenetic proteins with specific promoters, light can be used for cell type-specific stimulation. The most frequently used optogenetic protein is channelrhodopsin2, a light-gated non-selective cation channel. We have shown that optogenetic stimulation can be used to induce and prolong action potentials in channelrhodopsin2-expressing cardiomyocytes in vitro, and for atrial and ventricular pacing as well as arrhythmia induction in vivo. Optogenetic stimulation is a technology that combines the expression of light-sensitive proteins with stimulation of cells and organs by light, and can be used in vitro as well as in vivo. Optogenetic stimulation allows a very high spatio-temporal resolution because light can be focused to specific regions using lenses or light guides, and the exact time of activation and deactivation is defined by onset and duration of illumination. By expression of optogenetic proteins with specific promoters, light can be used for cell type-specific stimulation. The most frequently used optogenetic protein is channelrhodopsin2, a light-gated non-selective cation channel. We have shown that optogenetic stimulation can be used to induce and prolong action potentials in channelrhodopsin2-expressing cardiomyocytes in vitro, and for atrial and ventricular pacing as well as arrhythmia induction in vivo. For optical stimulation of G proteins, light-sensitive receptors are required. It has previously been demonstrated that a chimeric protein consisting of rhodopsin and the α1-adrenergic receptor (optoα1-AR) can be used for light-induced Gq activation and modulation of spike firing in neurons in vivo. Another light-activated Gq-coupled receptor is melanopsin, which is expressed in photosensitive ganglion cells of the retina and is involved in the regulation of circadian rhythm and pupillary light reflexes. Melanopsin was used before for optogenetic modulation of blood glucose homeostasis in diabetic mice by light-induced production and secretion of glucagon-like peptide 1 in artificial engineered and transplanted HEK293 cells.

The aim of this study was to show that optogenetic Gq activation can be performed in cardiomyocytes and investigated effects of light-induced Gq activation on spontaneous beating and local pacemaking. We found that melanopsin allows local and fast Gq activation with very low light intensities, and we believe that melanopsin is a useful new optogenetic tool for cardiovascular research.

2. Methods

An expanded Methods section is available in Supplementary material online.

2.1 Vector construction and analysis in HEK293FT cells

For construction of the CAG-melanopsin-IRES-green fluorescence protein (GFP) expression plasmid, melanopsin-IRES-GFP was excised from the pIRES2-OPN4AI plasmid (kindly provided by I. Provencio, University of Virginia) and cloned into an expression plasmid with the chicken β-actin (CAG) promoter as described before. Ca2+ imaging of transfected HEK293FT cells was performed with Rhod-2, and light-induced IP3 production was assessed by an immunoassay (Cisbio) for inositol-1-phosphate (IP1), a degradation product of IP3.

2.2 Embryonic stem cell generation and differentiation

G4 mouse embryonic stem (ES) cells were stably transfected with the CAG-melanopsin-IRES-GFP plasmid and differentiated in vitro in embryoid bodies (EBs) using the hanging drop method as reported earlier. To exclude unspecified light-induced effects, mouse ES cells and HEK293FT cells transfected with a plasmid for expression of the enhanced GFP under control of the chicken β-actin promoter (CAG-GFP) served as controls.
2.3 Analysis of pacemaking and optical stimulation

The frequency of spontaneously beating EBs was analysed by video microscopy as previously reported, using a dark setting and infrared light for imaging to avoid melanopsin activation by ambient or microscope light. For statistics, the change of frequency during illumination was normalized to baseline frequency before light application. To determine the localization of the leading pacemaker in a syncytium of cardiomyocytes, local field potentials were recorded with microelectrode arrays and conduction analysis was performed as described previously.

Cells were stimulated with blue light (470 nm) using a temperature-controlled LED module as described earlier, and light was attenuated with a neutral density filter.

2.4 Ca\(^{2+}\) imaging

Rhod-2 and Fura-2 were used for Ca\(^{2+}\) imaging experiments of HEK293FT cells and HL-1 cardiomyocytes, respectively, according to standard protocols. Because the excitation light already activated melanopsin, we used an imaging repetition rate as slow as possible to identify single Ca\(^{2+}\) transients and diastolic Ca\(^{2+}\) levels.

2.5 Immunohistochemistry and quantitative PCR

Immunostainings were performed according to standard protocols as reported earlier. Quantitative PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) and a Rotor-Gene 6000 quantitative PCR cycler (Corbett Life Science) as previously reported.

3. Results

3.1 Characterization of melanopsin function in HEK293FT cells

We chose melanopsin for light-induced activation of the G\(_q\)-signalling cascade (Figure 1A) because of the robust activation of G\(_q\) proteins by relatively low light intensities, compared with other optogenetic proteins. To identify melanopsin-expressing cells, we generated a new plasmid for co-expression of melanopsin and GFP with an internal ribosomal entry site under control of the CAG promoter (Figure 1B).

We have chosen this ubiquitous promoter, because it provides very high expression levels especially in muscle cells. To test the functionality of the generated construct, HEK293FT cells were transfected and melanopsin-expressing cells could be identified by GFP fluorescence. Melanopsin function was validated by measuring IP\(_1\), a degradation product of IP\(_3\), with an immunoassay in cells without and after 5 min of illumination (2.9 mW/mm\(^2\)). In the absence of retinal aldehydes, light-induced elevation of IP\(_1\) concentration could not be detected. However, after pre-treatment with 1 \(\mu\)mol/L of all-trans-retinal (ATR) or 11-cis-retinal, a robust light-induced increase of intracellular IP\(_1\) concentration could be observed (Figure 1C). We found that supplement of ATR was slightly more effective for light-induced IP\(_1\) increase than that of 11-cis-retinal (Figure 1C). Therefore, further experiments were performed after pre-treatment of cells with 1 \(\mu\)mol/L of ATR. Importantly, control HEK293FT cells exclusively expressing GFP did not show light-induced elevation of IP\(_1\) levels (Figure 1D).

To analyse intact downstream G\(_q\) signalling, Ca\(^{2+}\) imaging experiments were performed in melanopsin-expressing HEK293FT cells. In these experiments, repetitive light stimulation (10 s, 900 \(\mu\)W/mm\(^2\)) led to corresponding elevations of intracellular Ca\(^{2+}\) levels that dropped to baseline after termination of illumination (Figure 1E).

3.2 Generation of melanopsin-expressing cardiomyocytes

To obtain melanopsin-expressing cardiomyocytes, an ES cell line expressing melanopsin was generated by stable transfection of wild-type ES cells with the CAG-melanopsin-IRES-GFP plasmid (Figure 1B). Melanopsin transgenic ES cell colonies showed GFP fluorescence and immunostainings revealed melanopsin expression (Figure 2A). These ES cells

Figure 2 Functional melanopsin signalling in cardiomyocytes. (A) Immunostaining of a transgenic ES cell colony expressing melanopsin (left, red) and GFP (right, green). (B) Immunostaining of a differentiated EB with melanopsin (left, red) and GFP (right, green) expression in cardiac \(\alpha\)-actinin-positive (right, white) cardiomyocytes. (C and D) Representative frequency recordings of melanopsin-expressing EBs stimulated with light (C and D, blue bars, 308.5 nW/mm\(^2\)) or ET-1 (D, black bar, 100 nM). (E and F) Normalized acceleration of beating rate (E) and time to peak-frequency (F) of ET-1 and light stimulation (n = 4). Nuclei are shown in blue. Scale bars, 25 \(\mu\)m. Error bars, SEM. *p < 0.05, **p < 0.01.
were differentiated into cardiomyocytes by generating cell clusters, so-called EBs. Spontaneous beating of EBs was detected after Day 7, and experiments were performed at Days 8–9 of differentiation. At this early stage, the phenotype of ES cell-derived cardiomyocytes is relatively uniform and represents early embryonic pacemaker cells, whereas diversification into atrial and ventricular subtypes occurs later.\textsuperscript{26,27} Immunostainings showed clusters of cross-striated α-actinin-positive cardiomyocytes that were positive for GFP and melanopsin (Figure 2B).

Quantitative PCR revealed that melanopsin expression had no influence on mRNA expression levels of the α-subunit of G\textsubscript{q}(gnaq), PLC\textsubscript{b3} (plcb3) and B4 (plcb4), or IP\textsubscript{3} receptor Type 1 (itpr1) and Type 2 (itpr2) in ES cells (see Supplementary material online, Figure S1A) and differentiated EBs (see Supplementary material online, Figure S1B). Importantly, we observed differences neither in basal or ET-1-induced IP\textsubscript{3} levels (see Supplementary material online, Figure S1C) nor in spontaneous beating frequency (see Supplementary material online, Figure S1D) or ET-1-induced acceleration of beating rate (see Supplementary material online, Figure S1E) between melanopsin-expressing and control EBs.

### 3.3 Light-induced G\textsubscript{q} activation in cardiomyocytes

Brief illumination (60 s, 308.5 nW/mm\textsuperscript{2}) of spontaneously contracting EBs caused an instantaneous increase in beating frequency, which could be induced repetitively with similar responses (Figure 2C and Supplementary material online, Video S1). Illumination with the same light intensity did not influence spontaneous beating of control EBs (see Supplementary material online, Figure S2). Compared with pharmacological G\textsubscript{q} activation by ET-1 (60 s, 100 nM), the increase of beating rate induced by illumination (60 s, 308.5 nW/mm\textsuperscript{2}) was much stronger (212.8 ± 18.7 vs. 153.6 ± 3.3%, n = 4, P = 0.0032, Figure 2D and E) with faster reaching of peak frequencies (10.3 ± 1.9 vs. 21.2 ± 2.6 s, n = 4, P = 0.0017, Figure 2D and F). To prove that the observed increase in beating frequency was due to G\textsubscript{q} activation, we blocked different parts of the signalling cascade. Inhibition of PLC by U-73122 (10 μmol/L)\textsuperscript{28} had no effect on light-induced increase in beating frequency (253.6 ± 36.0 to 272.8 ± 40.0%, n = 3, P = 0.7575, Figure 3C).

To exclude a paracrine-mediated frequency increase due to light-induced release of hormones from neighbouring melanopsin-expressing cells, single cardiomyocytes were isolated from beating EBs and analysed. These cardiomyocytes were positive for GFP, melanopsin, and α-actinin (Figure 3D) and expressed the IP\textsubscript{3} receptor Type 2 (Figure 3E). Light application to single spontaneously contracting cardiomyocytes caused an increase in beating frequency similar to that observed in whole EBs (Figure 3F and Supplementary material online, Video SII), proving a direct effect of light-induced G\textsubscript{q} activation in cardiomyocytes.

To show melanopsin-induced Ca\textsuperscript{2+} release, Ca\textsuperscript{2+} imaging was performed with cardiomyocytes from the HL-1 cell line\textsuperscript{29} that were transfected to express melanopsin (Supplementary material online, Video SIII). Analysis of calcium transients showed a significant decrease of light-induced increase of beating frequency (10.3 ± 1.9 vs. 21.2 ± 2.6 s, n = 4, P = 0.0017, Figure 2D and F). Application of 2-aminoethoxydiphenyl borate (2-APB, 10 μmol/L)\textsuperscript{28} to block IP\textsubscript{3} receptors and canonical transient receptor potential channels reduced the light-evoked increase of beating rate from 239.4 ± 30.2 to 129.6 ± 17.6% (n = 6, P = 0.0078, Figure 3B and C). Blocking of ryanodine receptors with ryanodine (10 μmol/L)\textsuperscript{28} had no effect on light-induced increase in beating frequency (253.6 ± 36.0 to 272.8 ± 40.0%, n = 3, P = 0.7575, Figure 3C).

Figure 3 Validation of the melanopsin signalling pathway in cardiomyocytes. (A and B) Representative frequency recordings of melanopsin-expressing EBs in the presence of 10 μmol/L of U-73122 (A) or 10 μmol/L of 2-APB (B). Light stimulation (111.5 nW/mm\textsuperscript{2}) indicated by top blue bars, and application of blockers indicated by red bars. (C) Statistical analysis of normalized mean frequency during illumination was performed with cardiomyocytes from the HL-1 cell line\textsuperscript{29} that were transfected to express melanopsin (Supplementary material online, Video SIII).
3.4 Relationship between melanopsin signalling and light intensity

To analyse the dependence of acceleration in beating rate on light intensity, we stimulated beating EBs with stepwise increasing light intensities from 1.8 to 308.5 nW/mm² (60 s, n = 6–7). Increasing light intensities led to more pronounced elevations in beating frequency (Figure 4A and B). To account for variations in beating frequencies between EBs, the highest peak frequency upon illumination and mean plateau frequency during the last 30 s of light stimulation were normalized to baseline frequency before light application. This analysis showed that illumination with low light intensity (9.1 nW/mm²) led to a small and delayed elevation of beating frequency to 134.2 ± 7.2% of baseline. Stimulation with high light intensity (308.5 nW/mm²) resulted in an instantaneous frequency increase to 317.1 ± 16.3%, which subsequently declined with a time constant of 14.1 ± 3.1 s (n = 6) to a constant plateau frequency of 210.0 ± 15.0% (Figure 4B and C).

In analogy to dose–response relationships of receptor activation with agonists, we plotted the normalized peak and plateau frequencies against the logarithmic light intensity. This resulted in sigmoid light intensity–response relationships that could be fitted with Hill equations (Figure 4C) to determine the half maximal effective light intensity (EL50). We found an EL50 of 41.7 nW/mm² when analysing peak frequency and an EL50 of 29.8 nW/mm² for plateau frequency. To quantify the peak-to-plateau relationship, we calculated the ratio between peak and plateau frequency for each individual experiment. Interestingly, this analysis showed that a clear peak in frequency with decline to plateau levels could only be observed at light intensities above ~30 nW/mm² (Figure 4D).

3.5 Temporal aspects of melanopsin signalling

Because using light allows instantaneous receptor activation and deactivation, we were able to calculate precise activation and deactivation kinetics of frequency modulation by Gq signalling. Using low light intensity (9.1 nW/mm²), the fastest beating rate was reached after 42.4 ± 5.5 s, whereas the delay was only 10.2 ± 1.7 s when saturating light intensity (308.5 nW/mm²) was applied. This delay from switching on the light to reaching peak frequency depended on the applied light intensity with an EL50 of 42.9 nW/mm², and that irregular beating only occurred after light intensities above ~30 nW/mm² (Figure 5C). There seems to be a correlation between peak frequency and the occurrence of irregular beating because plotting the peak frequency of each light intensity vs. the corresponding arrhythmia index (CV) showed a linear relationship with a correlation coefficient R² of 0.91 (Figure 5D).

3.6 Irregular beating after melanopsin activation

Optical Gq activation by high light intensity stimulation was followed by irregular beating in the form of abrupt accelerations of beating rate, prolonged pauses, and bigeminal rhythms (continuous alternation of short and long cycles) (Figure 5A). Interestingly, this occurred after termination of illumination and persisted for up to 4 min. To quantify irregular beating, we analysed the coefficient of variation (CV) of interbeat intervals after termination of illumination (Figure 5B) (for details see Supplementary material online, methods). We found that this value depended on the applied light intensity with an EL50 of 42.9 nW/mm², and that irregular beating only occurred after light intensities above ~30 nW/mm² (Figure 5C). There seems to be a correlation between peak frequency and the occurrence of irregular beating because plotting the peak frequency of each light intensity vs. the corresponding arrhythmia index (CV) showed a linear relationship with a correlation coefficient R² of 0.91 (Figure 5D).

3.7 Local stimulation of melanopsin

Local activation of Gq signalling using receptor agonists is technically challenging because of diffusion after application. In contrast, optogenetic Gq activation by focused light enables locally restricted Gq stimulation. To investigate the relevance of spatially confined Gq activation, whole EBs were plated on microelectrode arrays that allow recording of local field potentials from beating cardiomyocytes (Figure 6A). Latency analysis of field potentials was used to identify the leading pacemaker site during spontaneous beating and the direction of conduction within the syncytium of electrically coupled cardiomyocytes (Figure 6B). Illumination of a small area (Ø 650 μm, 308.5 nW/mm²) opposite of the leading pacemaker site caused an increase in beating frequency (Figure 6A, enlargement). Importantly, latency analysis showed that, during illumination, pacemaking was initiated at the illuminated site and electrical activity was conducted to non-illuminated areas (Figure 6C).

4. Discussion

The aim of this study was to establish melanopsin as a new optogenetic tool in cardiovascular research and to use it to investigate the physiological role of Gq signalling in pacemaking of cardiomyocytes at an early developmental stage. The advantages of optogenetic approaches are manifold because they allow non-contact stimulation of a specific cell type with down to millisecond and micron-scale resolution.

4.1 Side effects of melanopsin overexpression

Overexpression of light-sensitive proteins might modulate or disturb the native intracellular signalling machinery even without illumination because of protein binding or other overexpression artefacts. Therefore, we have analysed the gene expression levels of major components
of the $G_q$-signalling cascade, the baseline and agonist-induced IP$_3$ production, as well as the spontaneous and agonist-stimulated beating rates. We found that all these parameters were similar in melanopsin and control ES cells and EBs. Because we did not analyse protein expression, we cannot fully exclude minor side effects on the native $G_q$-signalling cascade by overexpression of exogenous melanopsin. However, this is unlikely because baseline frequency and agonist-induced modulation of pacemaking are not altered by melanopsin expression.

4.2 Modulation of pacemaking by light-induced $G_q$ stimulation

Ca$^{2+}$ oscillations are involved in the regulation of pacemaking in cardiomyocytes derived from early embryonic hearts$^{6,9,10}$ or from ES cells.$^7$ These Ca$^{2+}$ oscillations originate from intracellular stores and are translated into membrane depolarization by the sodium–calcium exchanger leading to action potential generation and spontaneous beating.$^6,9$ Hyperpolarization-activated cyclic nucleotide (HCN)-gated channels

**Figure 4** Dependency of melanopsin activation on light intensity and light-pulse duration. (A) Representative frequency recording of a melanopsin-expressing EB stimulated with stepwise increasing light intensities (blue trace, 60 s). (B) Overlay of frequencies from (A) in response to different light intensities (blue bar), note the peak and plateau phase at higher intensities. (C) Light intensity—response relationship for normalized peak (maximum) and plateau (mean of last 30 s) frequency fitted with Hill equations ($n = 6–7$). (D) Relationship between the ratio of peak-to-plateau frequency and light intensity fitted with a Hill equation ($n = 6–7$). (E) Relationship of time to peak and light intensity fitted with a Hill equation ($n = 6–7$). (F) Overlay of beating frequencies in response to different light-pulse durations (duration indicated by bar in matched colour, 308.5 nW/mm$^2$). (G) Light-pulse duration—response relationship for normalized peak frequency fitted with a Hill equation ($n = 3–6$). (H) Recovery from inactivation for two different light intensities with interpulse intervals of 10 (black) and 90 s (grey) ($n = 3–4$) (for details see text and Supplementary material online, methods). Error bars, SEM. **$P$, $0.01$. T. Beiert et al. 512 Downloaded from https://academic.oup.com/cardiovascres/article-abstract/102/3/507/349771 by guest on 12 January 2018
play a minor role at this early stage, because their blockage has no or little effect on beating frequencies in vitro\(^7,9\) and blockage or genetic ablation of HCN channels does not completely inhibit spontaneous beating of early (E9.5) hearts in vivo\(^30\).

In this study, we analysed EBs on Days 8–9 of differentiation in which cardiomyocytes are in an early developmental stage with a relatively uniform early pacemaker-like phenotype.\(^{26,27}\) Light-induced G\(_q\) activation increased the firing rate of spontaneously beating EBs. This effect was highly specific to melanopsin and G\(_q\) signalling, because it was not observed in control cardiomyocytes and it was attenuated by blockage of components of the G\(_q\)-signalling cascade. Using slowly beating HL-1 cardiomyocytes, we were able to demonstrate light-induced elevation of diastolic Ca\(^{2+}\) that further supports G\(_q\)-induced Ca\(^{2+}\) release from IP\(_3\)-sensitive stores. Paracrine effects through liberation of hormones from neighbouring cells in the multicellular EB could be excluded because isolated single ES cell-derived cardiomyocytes showed a similar light-induced frequency increase.

We conclude that light-induced stimulation of melanopsin induces PLC activity and IP\(_3\) generation that leads to Ca\(^{2+}\) release by IP\(_3\) receptors in early ES cell-derived cardiomyocytes. This, in turn, enhances spontaneous Ca\(^{2+}\) oscillations and thereby increases spontaneous pacemaking activity. This is fully in line with previous reports, which showed reduced or abolished beating in the presence of the IP\(_3\) receptor antagonists Xestospongin C\(^7\) and 2-APB\(^6,8,9\) as well as the PLC blocker U-73122.\(^8\) In addition, G\(_q\)-coupled agonists like phenylephrine and ET-1\(^8,10\) or direct administration of a membrane permeable form of IP\(_3\)\(^9\) caused an increase in beating frequency in embryonic cardiomyocytes. This principle also seems to be important during embryonic pacemaking in vivo because embryonic hearts at very early developmental stages show high levels of endogenous ET-1 and the blockage of ET receptors induces bradycardic arrhythmia in utero.\(^10\) Since cells of the sinus node express IP\(_3\) receptors,\(^6\) G\(_q\) activation could influence pacemaking also in the adult heart. Furthermore, the G\(_q\)-coupled agonist ET-1 is known to induce arrhythmogenic Ca\(^{2+}\) responses in ventricular and atrial cardiomyocytes,\(^13,14\) which were abolished in cardiomyocytes from IP\(_3\) receptor 2 knockout mice.\(^14\) Because expression of IP\(_3\) receptors is up-regulated in cardiac disease, G\(_q\)-induced Ca\(^{2+}\) release becomes more important in such pathological states.\(^5\) To investigate

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**Figure S** Melanopsin activation induces irregular beating. (A) Representative frequency analysis of a melanopsin-expressing EB stimulated with 39.4, 69.1, and 111.5 nW/mm\(^2\) (indicated by top black bars). Enlargement of the framed region shows individual beats by vertical lines during and after 111.5 nW/mm\(^2\) stimulation (black bar). Note the irregular beating after termination of illumination. (B) CV of interbeat intervals after termination of illumination with the indicated light intensity. Significance tested against CV without illumination (n = 7). (C) Relationship between arrhythmic index (represented by CV from B) and light intensity fitted with a Hill equation (n = 7). (D) Arrhythmic index (CV) plotted against the normalized peak frequency during illumination with a linear fit (n = 6–7) (for details see text and Supplementary material online, Methods). Error bars, SEM. *P < 0.05. **P < 0.01.
of highly overexpressed melanopsin stimulates the G_q-signalling pathway to a larger and eventually non-physiological extent than using an endogenous receptor. Therefore, transferring this observation to the pathophysiology of arrhythmias has to be done with caution.

However, we speculate that overexpression of an optogenetic G_q protein-coupled receptor might be useful to ‘simulate’ effects of enhanced receptor expression and higher activity of G_q signalling, which is known to occur in cardiac pathology.5

4.4 Temporal aspects and inactivation of light-induced G_q signalling
Illumination of melanopsin allows repetitive and instantaneous on- and off-switching of the G_q-signalling pathway, which cannot be performed with agonists with similar temporal precision. We used this to determine the activation and deactivation kinetics of the G_q-signalling cascade in pacemaking of cardiomyocytes. Using saturating light intensity, we found that an increase in beating frequency started almost instantaneously, peak frequency was reached within ~10 s and light-pulse durations as short as ~5 s were sufficient for maximal response. Interestingly, high-intensity stimulation led to a biphasic frequency response with a peak frequency that inactivated to a sustained plateau frequency. This inactivation affects subsequent stimulations and recovers with a time constant of ~50 s. In contrast, low intensity stimulation does neither generate a biphasic response nor induce prolonged inactivation. The mechanism for the high light intensity-induced inactivation can be explained by either (i) desensitization of melanopsin, (ii) negative feedback and inactivation of various parts of the G_q-signalling cascade (‘biochemical adaptation’), or (iii) reduction of IP3 receptor activity by elevated Ca2+ levels because of the bell-shaped dependence on intracellular Ca2+ concentrations.31 The observation that peak frequency with inactivation as well as prolonged irregular beating occurs only at high-intensity stimulation suggests a similar mechanism underlying both effects. This assumption makes a negative feedback on the G_q-signalling cascade the more likely explanation because after termination of illumination desensitized melanopsin does not interfere with pacemaking and Ca2+ has returned to basal levels. This interpretation is in line with the proposed mechanism (‘biochemical adaptation’) for desensitization and light adaption of the melanopsin-based phototransduction cascade of intrinsic photosensitive retinal ganglion cells.32

4.5 Dose–response relationships and light sensitivity
Generation of dose–response relationships using receptor agonists requires precise agonist concentrations at the cells of interest which depends on perfusion and diffusion characteristics, especially in multicellular preparations. We found that light-induced frequency increase was highly reproducible even after repetitive stimulations. This allowed accurate stimulation with various light intensities and thereby a straightforward analysis of dose–response relationships.

In analogy to the half maximal effective concentration for receptor agonists, we defined EL50 as the half maximal effective light intensity for a specific effect. We found that EL50 was only 41.7 and 29.8 nW/mm² for reaching peak and plateau frequencies, respectively. These values are 10^3–10^4 times lower than the light intensities required for optogenetic stimulation of channelrhodopsin2 in cardiomyocytes (~1 mW/mm²).16 The high light sensitivity of melanopsin poses some disadvantages in general handling of cells and performing of experiments, but also has great advantages especially when used in vivo. For detailed discussion, see technical note in Supplementary material online.

Figure 6 Confined melanopsin activation induces local pacemaking. (A) Field potentials from cardiomyocytes within an EB plated on a microelectrode array. Blue dot marks the centre of local illumination. Time of local illumination (308.5 nW/mm²) is indicated by blue bar in the enlargement of a single electrode. (B and C) Activation maps before (B) and during (C) illumination with colour-coded latency and isochronal lines 10 ms apart. Area of illumination (Ø 650 μm) is indicated by blue circle (C).
4.6 Spatial resolution

We have shown that focused light can be used for locally confined Gq activation within a functional syncytium of melanopsin-expressing cardiomyocytes, and that this is sufficient to induce local pacemaking. Thus, at the early developmental stage investigated here, the levels of Gq activity seem to be a potent factor for determining the leading pacemaker. Localized Gq activation cannot be achieved with pharmacological agonists because they diffuse and thereby make determination of exact borders of stimulation impossible. Being able to locally activate the Gq cascade might be very useful to investigate the location of Gq-sensitive cardiomyocytes in the developing heart in vivo, or to investigate IP3-dependent communication via gap junctions between endothelial and/or smooth muscle cells in the vascular wall.

4.7 Outlook and potential applications in vivo

Channelrhodopsin2 and other optogenetic tools have been extensively used for in vivo applications in the brain but also in the heart.16,17 One important feature of optogenetics is the possibility of cell type-specific expression when using specific promoters. Melanopsin, therefore, enables investigation of cell type-specific effects of Gq-signalling cascade in the cardiovascular system with high spatial and temporal resolution in vitro and in vivo. Channelrhodopsin2 and other optogenetic tools have been extensively used to study the importance of Gq signalling in cardiac pacemaking in vivo. In such experiments, cell-specific expression in cardiomyocytes would exclude side effects of systemically applied Gq-coupled receptor agonists on endothelial and smooth muscle cells that might influence coronary perfusion. Taken together, optogenetic Gq activation corroborates the importance of the Gq-signalling cascade in pacemaking in cardiomyocytes. Furthermore, melanopsin transgenic mice could be used to study the importance of Gq signalling in cardiac pacemaking in vivo. In such experiments, cell-specific expression in cardiomyocytes would exclude side effects of systemically applied Gq-coupled receptor agonists on endothelial and smooth muscle cells that might influence coronary perfusion.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank F. Holst (University of Bonn) for technical assistance, H. Begerau (University of Bonn) for designing and programming the frequency analysis software, I. Provenzio (University of Virginia) for providing the pIRES2-OPN4A1 plasmid, F. Polleux (Scripps Research Institute) for providing the pCIG2 plasmid, and A. Nagy (Mount Sinai Hospital, Toronto) and M. Gertsenstein (Mount Sinai Hospital, Toronto) for providing the G4 mouse ES cell line.

Conflict of interest: none declared.

Funding

This work was supported by the Bonfor program (Medical Faculty, University Bonn), the German Heart Foundation, and the German Research Foundation (grant [FL 276/3-3], Research Training Group 1873 and Research Unit 917 ‘Nanoparticle-based targeting of gene- and cell-based therapies’ (SA 1785/5-1 and FL 276/6-1)).

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