Alterations of field potentials in isotropic cardiomyocyte cell layers induced by multiple endogenous pacemakers under normal and hypothermal conditions

R. Kienast, M. Stöger, M. Handler, F. Hanser, and C. Baumgartner

1Institute of Electrical and Biomedical Engineering, University for Health Sciences, Medical Informatics and Technology, Hall in Tyrol, Austria; and 2Division of Internal Medicine III/Cardiology, Medical University Innsbruck, Innsbruck, Austria

Submitted 11 February 2014; accepted in final form 29 July 2014

Kienast R, Stöger M, Handler M, Hanser F, Baumgartner C. Alterations of field potentials in isotropic cardiomyocyte cell layers induced by multiple endogenous pacemakers under normal and hypothermal conditions. Am J Physiol Heart Circ Physiol 307: H1013–H1023, 2014. First published August 1, 2014; doi:10.1152/ajpheart.00097.2014.—The use of autonomous contracting randomly grown cardiomyocyte monolayers cultivated on microelectrode arrays (MEAs) represents an accepted experimental setting for preclinical experimental research in the field of cardiac electrophysiology. A dominant pacemaker forces a monolayer to adhere to a regular and synchronized contraction. Randomly distributed multiple pacemakers interfere with this dominant center, resulting in more or less frequent changes of propagation direction. This study aims to characterize the impact of changing propagation directions at single electrodes of the MEA on the four intrinsic parameters of registered field potentials (FPs) FPan, FPMIN, FPpre, and FPdune and conduction velocity (CV) under normal and hypothermal conditions. Primary cultures of chicken cardiomyocytes (n = 18) were plated directly onto MEAs and FPs were recorded in a temperature range between 37 and 29°C. The number and spatiotemporal distribution of biological and artificial pacemakers of each cell layer inside and outside of the MEA registration area were evaluated using an algorithm developed in-house. In almost every second myocardial cell layer, interfering autonomous pacemakers were detected at stable temperatures, showing random spatial distributions with similar beating rates. Additionally, a temperature-dependent change of the dominant pacemaker center was observed in n = 16 experiments. A significant spread-direction-dependent variation of CV, FPan, FPMIN, and FPpre up to 14% could be measured between different endogenous pacemakers. In conclusion, based on our results, disregarding the spatial origin of excitation may lead to misinterpretations and erroneous conclusions of FP parameters in the verification of research hypotheses in cellular electrocardiology.

microelectrode array; field potentials; hypothermia; spread-direction dependency; myocardial cells

Address for reprint requests and other correspondence: R. Kienast, UMIT-Univ. for Health Sciences, Medical Informatics and Technology, Institute of Electrical and Biomedical Engineering, Eduard Wallnöfer-Zentrum 1, 6060 Hall in Tyrol, Austria 2014-02-05 (e-mail: roland.kienast@umit.at).

http://www.ajpheart.org

0363-6135/14 Copyright © 2014 the American Physiological Society
ALTERATIONS OF FIELD POTENTIALS IN CARDIOMYOCYTES

The myocardial cell layer with multiple active pacemaker centers outside the micro-electrode arrays (MEA) registration area. At first appearance the morphological structure of the cell layer seems to be homogeneous. Considering the substructural cell organization (see zoomed areas) randomly grown cardiomyocytes formed locally an anisotropic but not predictable structure (in terms of cell density and orientation, cell size, distribution of gap junctions, and their conductance). See also Fig. 3.

Fig. 1. A schematic illustration of a randomly grown “quasi-isotropic” myocardial cell layer with multiple active pacemaker centers outside the micro-electrode arrays (MEA) registration area. At first appearance the morphological structure of the cell layer seems to be homogeneous. Considering the substructural cell organization (see zoomed areas) randomly grown cardiomyocytes formed locally an anisotropic but not predictable structure (in terms of cell density and orientation, cell size, distribution of gap junctions, and their conductance). See also Fig. 3.

upstroke velocity (17). However, this observation can lead to misinterpretations in signal analysis when, for example, studying the characteristics of upstroke velocity at different temperatures or after pharmacological stimuli as also hypothesized in the work of Sommerhage et al. (47).

Therefore, the investigation of electrophysiological alterations in quasi-isotropic cell cultures (including multiple pacemaker centers) under normal and/or hypothermal conditions leads to the following scientific question:

Is there evidence of excitation direction changes and alterations of extracellularly recorded FPs in quasi-isotropic myocardial cell layers registered at single electrodes of the MEA under normal and hypothermal conditions?

To tackle this question detailed information about spatial distribution and temporal activation of pacemaker centers and related changes of wavefront propagation direction is needed to study the impact of the spatial origin of excitation on selected intrinsic FP features under hypothermal conditions. One approach is to use artificial stimulation with well-defined stimulation parameters for time and position. Compared with autonomous pacemakers, artificial pacing may, however, lead to unexpected changes of electrophysiological parameters such as the CV (40). To investigate these mechanisms a new algorithm was developed to compute spatial and temporal distribution of biological and artificial excitation sources located inside and outside the MEA registration area. Through the use of this approach we are able to demonstrate that CVs and intrinsic parameters of FPs in quasi-isotropic myocardial cell prepara-

Fig. 2. A: spatial distribution of pacemakers in the myocardial cell layer where number 2 indicates an artificial stimulus. B: boxplot of the spread-dependent field potential rise (FP\text{rise}) time for the 3 different pacemaker (2 biological and 1 artificial) centers in a single cell layer at 35°C measured at a single electrode. C: yields the same data as in B over time. Physiologically unexpected jumps in data caused by alternations of the pacemaker centers are evident. The rectangle in A indicates the active MEA registration area.

METHODS

Cell cultivation. In this study we analyzed intrinsic parameters of FPs for n = 18 primary cultures of chicken cardiomyocytes. As previously described in Ref. 25, the hearts of 12-day-old chicken embryos were extracted and the ventricles were separated from the atrium. The isolated ventricles were minced and digested with 0.05% trypsin. One drop of the obtained cell suspension was platted onto the fibronectin-coated MEA covering the electrode grid located in the center of a Petri dish. One to two minutes thereafter the Petri dish was carefully filled with 1.5 ml of culture medium. One milliliter of medium was renewed daily. After 3–4 days of incubation, the cells formed a firmly attached and spontaneous beating monolayer covering the whole electrode area that was used for MEA recordings (Fig. 3). After registration of the FPs under hypothermal conditions, each cell preparation was ineligible for further measurements and discarded.

MEA recording. Eighteen planar MEAs (Multi Channel Systems, Reutlingen, Germany) with three different electrode configurations and an integrated reference electrode placed on the bottom of the
proximately the dominant biological pacemaker’s frequency (ap-
approximately 830,000 separately measured FPs at the different temperature levels of 37, 35, 33, 31, and 29°C. For artificial pacing, two record-
ners were used. In our experiments with chicken cardiomyocytes at
37°C, we only could observe a small depolarization wave roughly 210
ms after FP_{MIN} with a mean amplitude of 12 μV. Analogous time
parameters for repolarization, also measured for chicken cardiom-
yocytes, were described by Krogh-Madsen et al. (31). This small
depolarization wave is apparent if the registered signals yield a stable baseline with a small noise component. However, the poor signal-to-
noise ratio relative to FP_{MAX} somewhat limited the detection accuracy of the depolarization wave and consequently the FP_{dur} time in our
experiments.

In addition to the previously described FP features, differences of
CV in single electrodes were determined depending on the origin of
excitation. Analogous to previously introduced methods (4, 37), CV
fields registered from in vitro experiments were calculated from the
two-dimensional spatial gradient of the waveform arrival time T(x,y).
The gradient of a single waveform moving across the MEA was
calculated numerically, exactly at the position of the electrodes. This
implies that the local coordinates x and y are held constant and only
activation time t changes. Therefore, the velocity vector \( \mathbf{v} = [dx/dt, dy/dt] \)
of each single electrode can be simply computed by inverting the
elements of the gradient \( \text{grad} T = [\partial T / \partial x, \partial T / \partial y] \) to obtain the velocity
vector field \( \mathbf{V}(x,y,t) \). It is well known that decreasing temperature has a
strong impact on cardiac electrophysiology such as prolonged
depolarization time and decreased CV and firing rate of pacemaker
cells. However, in this study we investigated the influence of spread
direction of multiple endogenous pacemakers on intrinsic FP features
and CV by analyzing FP morphologies at stable temperature levels of
37, 35, 33, 31, and 29°C to exclude side effects during temperature
changes.

**Data analysis.** The data were analyzed offline with an in-house
devolved software tool based on MATLAB (The Mathworks, Natick,
MA) to detect and characterize FP features and CV. To statistically
evaluate direction-dependent effects of the four FP features \( \text{FP}_{\text{rise}}, \text{FP}_{\text{MIN}}, \text{FP}_{\text{pre}}, \) and \( \text{FP}_{\text{dur}} \) and CV at single electrodes of the MEA, first
a Shapiro-Wilk test was applied to test whether the data follows a
normal distribution \( (P < 0.05) \). Since most of the data is not normally
distributed, a Kruskal-Wallis one-way analysis was employed to
calculate the values of the respective feature for two or more different
pacemaker cells at different temperature levels for statistical sig-
nificance (nonparametric multiple class testing). Differences were
considered statistically significant for \( P < 0.01 \) using Bonferroni
correction for multiple Kruskal-Wallis one-way testing. Due to the

**Table 1. Electrode configurations for FP registrations**

<table>
<thead>
<tr>
<th>Electrode Diameter, μm</th>
<th>Electrode Material</th>
<th>Interelectrode Distance, μm</th>
<th>Electrode Layout</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>TiN</td>
<td>200</td>
<td>grid, 8 x 8</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>TiN</td>
<td>200</td>
<td>grid, 8 x 8</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>Gold</td>
<td>700</td>
<td>grid, 8 x 8</td>
<td>3</td>
</tr>
</tbody>
</table>

FP, field potential; TiN, titanium nitride; n, number of performed experi-
mental studies with the same electrode configuration.
unpredictable number and various beat intervals of active pacemaker centers, the number of Kruskal-Wallis tests differs between the individual temperature levels. However, to obtain a comparable representation, results are shown as bar charts normalized to 100% and the number of the performed Kruskal-Wallis tests is denoted in the respective bar as additional information. Due to altered morphology of the different myocardial cell preparation, distinct electrode-cell-coupling, and temperature, absolute values of investigated features vary. To illustrate the impact of various propagation directions, relative changes of FP features between the pacemaker center with the lowest and highest values are calculated. Because of predominantly left skewed data distributions, these changes are denoted by the median and the median absolute deviation (MAD).

RESULTS

**Absolute values of intrinsic FP features and CV.** Table 2 shows mean values of CV and investigated intrinsic FP features determined from our experimental data. The registered signal amplitude of $F_{\text{pre}}$ and $F_{\text{MIN}}$ depends on the selected electrode array. All other features show no significant differences between the used electrode configurations. In addition, well known hypothermia-induced effects such as a decrease in CV or an increase in AP duration ($F_{\text{dur}}$) can be observed.

**Pacemaker centers.** As described in Refs. 10 and 21, a myocardial cell culture forms weakly coupled islets of cell aggregates with individual active pacemakers in the first days of incubation. After 3–4 days, these islets merge to a continuous cell layer and a dominant center emerges. This center forces the layer to a synchronized, stable, and regular contraction, more or less interfering with other randomly distributed pacemakers where the contraction frequency depends on the initial seeding density and the age of the study animal. The phenomenon of multiple pacemaker centers in a culture has been thoroughly discussed for embryonic stem cells of line D3 (mouse) (22) for primary cultures of rat ventricular myocytes (15, 47) and murine atrial cardiomyocyte-derived cell line HL-1 (30). In our experimental data, multiple autonomous (biological) pacemaker centers were detected in almost every second myocardial cell layer at different stable temperature levels of 37, 35, 33, 31, and 29°C (Fig. 6). As also described by Feld et al. (15) and confirmed by our observations, in most cases pacemaker centers formed outside the MEA registration area. Table 3 gives detailed information about the frequency of all active pacemaker centers and their average counts with standard deviations in mild and moderate hypothermia. The beating rates of active pacemakers show stable frequencies of about 0.5 Hz (30 beats/min) at 29°C to ~1.5 Hz (90 beats/min) at 37°C (Fig. 7A). The beating rates of interfering pacemaker centers are in a similar range where the frequency variation is in most of the experiments <0.1 Hz (6 beats/min; Fig. 7B). In addition to the interference of the dominant pacemaker center by other pacemakers at stable temperatures, we also observed a temperature-dependent alteration of propagation direction that led to changes of the dominant pacemaker region in the cell layer during cooling in almost all analyzed myocardial cell cultures [$n = 16$ of total $n = 18$; a link to movies, visualizing changes in excitation direction is provided in the APPENDIX (see Links to raw data and movies)]. However, the number of active pacemaker centers at the prevailing temperature and the position of the dominant center differ from experiment to experiment. In our cell preparations 4.0 (SD 1.9) centers on average...
were detected in the considered temperature range from 37 to 29°C. Figure 8 illustrates time series experiments of active pacemaker centers and their spatial distributions for two cell layers. Temperature-induced changes of the dominant pacemaker center can be observed in both cell layers.

Conduction velocity. Figure 9A depicts a boxplot of calculated CV of two autonomous pacemaker centers from the same electrode at different temperatures. The well-known effect of CV reduction with decreasing temperature is evident. Statistical analysis of CV for all experimental datasets using a Kruskal-Wallis test, however, yields a significant correlation between CV and propagation direction at stable temperatures (Fig. 10A) with a median difference of 6% (MAD = 4) between different pacemakers.

\( F_{\text{rise}} \). The spread dependency of \( F_{\text{rise}} \) time measured at single electrodes could be experimentally confirmed for different temperatures. Figure 9B shows a boxplot demonstrating a correlation of \( F_{\text{rise}} \) and propagation direction for active pacemaker centers at the same electrode position. The bar graph in Fig. 10B summarizes the results of multiple testing for all experiments in the given temperature range from 37 to 29°C. The median change of \( F_{\text{rise}} \) between different propagation directions of multiple pacemakers is ~14% (MAD = 8).

Furthermore, Halbach et al. (17) and Banach et al. (3) observed a distance-dependent behavior of \( F_{\text{rise}} \) with respect to the location of a given pacemaker center. In this study we were not able to confirm a significant relationship between \( F_{\text{rise}} \) and the distance to the pacemaker centers (see Distance-dependent behavior of \( F_{\text{rise}} \) in the APPENDIX).

\( F_{\text{MIN}} \). As known, there is a significant relationship between \( F_{\text{MIN}} \) and \( Na^+ \) current (17). Again Kruskal-Wallis testing was performed (Fig. 10C), confirming a spread-direction dependency of the parameter \( F_{\text{MIN}} \) with a median change of ~13% (MAD = 8) as shown exemplarily in Fig. 9C for a single experiment.

\( F_{\text{pre}} \). Halbach et al. (17) described \( F_{\text{pre}} \) as a contribution of neighboring, previously excited tissue. Interestingly, based on the correlation between \( F_{\text{MIN}} \) amplitude and temperature (see \( F_{\text{MIN}} \) in RESULTS) our experiments also yielded a decrease in the amplitude of \( F_{\text{pre}} \) with decreasing temperatures. In addition, we observed a propagation direction dependency of \( F_{\text{pre}} \) that could be statistically confirmed by multiple testing (Figs. 9D and 10D) for all considered temperature levels. The determined \( F_{\text{pre}} \) values vary by ~13% (MAD = 8) between different excitation directions.

\( F_{\text{dur}} \). \( F_{\text{dur}} \) is defined by the time between \( F_{\text{MIN}} \) and \( F_{\text{MAX}} \) and shows a relationship to AP duration (17). Similar to the repolarization time of chicken cardiomyocytes as described by Krogh-Madsen et al. (31), \( F_{\text{MAX}} \) was measured ~240 ms after \( F_{\text{MIN}} \) at 37°C. After visual inspection of our experimental data and according to the literature (16, 26), \( F_{\text{dur}} \) is estimated to be in the range between 300 and 500 ms at the lowest considered temperature level of 29°C. Based on the fact that AP duration increases with decreasing temperatures (1, 26), \( F_{\text{dur}} \) can, therefore, be expected in the range of 240 to 500 ms. Due to the poor signal-to-noise ratio relative to \( F_{\text{MAX}} \), the detection accuracy of \( F_{\text{dur}} \) is, however, somewhat limited. This leads to some false detections of \( F_{\text{dur}} \) outside the expected physiological range. As a consequence, \( F_{\text{dur}} \) data beyond the range of 100 to 800 ms were removed before Kruskal-Wallis testing. Nevertheless, a direction dependency of \( F_{\text{dur}} \) could not be obviously confirmed at the single electrodes (Figs. 9E and 10E). The averaged difference between varying excitation directions is, however, not >1%.

Impact of artificial stimuli on CV and intrinsic FP parameters. As aforementioned, in \( n = 13 \) myocardial cell layers an artificial stimulus was set in the middle right center of the MEA. We observed a stronger impact on the FP morphology and CV for artificial stimuli compared with biologically

Table 2. Mean values of investigated FP features at different temperatures

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>CV, cm/s</th>
<th>( F_{\text{rise}} ), ms</th>
<th>( F_{\text{pre}} ), mV</th>
<th>( F_{\text{dur}} ), ms</th>
<th>( F_{\text{MIN}} ), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>20.4</td>
<td>0.194</td>
<td>0.51</td>
<td>1.40</td>
<td>1.14</td>
</tr>
<tr>
<td>31</td>
<td>22.3</td>
<td>0.153</td>
<td>0.57</td>
<td>1.37</td>
<td>1.14</td>
</tr>
<tr>
<td>33</td>
<td>24.0</td>
<td>0.155</td>
<td>0.58</td>
<td>1.60</td>
<td>1.22</td>
</tr>
<tr>
<td>35</td>
<td>26.0</td>
<td>0.137</td>
<td>0.57</td>
<td>1.71</td>
<td>1.50</td>
</tr>
<tr>
<td>37</td>
<td>27.8</td>
<td>0.138</td>
<td>0.62</td>
<td>1.89</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Table 3. Myocardial cell cultures with more than 1 active pacemaker center at temperatures of 29, 31, 33, 35, and 37°C

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>( n \geq 2 ) Active Pacemakers/Total Number of Cell Layers</th>
<th>No. of Active Pacemakers (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>7/12</td>
<td>2.29 (SD 0.49)</td>
</tr>
<tr>
<td>31</td>
<td>9/17</td>
<td>2.44 (SD 0.53)</td>
</tr>
<tr>
<td>33</td>
<td>6/18</td>
<td>2.17 (SD 0.41)</td>
</tr>
<tr>
<td>35</td>
<td>9/18</td>
<td>3.00 (SD 0.87)</td>
</tr>
<tr>
<td>37</td>
<td>10/18</td>
<td>2.70 (SD 0.95)</td>
</tr>
</tbody>
</table>

In the majority of cell layers 2 or 3 centers were active.
autonomous pacemakers (see Impact of artificial stimuli on CV and intrinsic FP parameters in the Appendix).

DISCUSSION

Recording FPs using MEAs is a common tool for investigating various scientific questions in the field of cardiac electrocardiography, for example, in vitro pharmacological stimulation and testing. The correlation between time and shape features of the AP and FP invites us to draw conclusions regarding the electrophysiological mechanisms on cardiomyocyte cell layers during cooling. Excitation-dependent features such as CV are, however, difficult to detect from recorded signals in unorganized homogeneous cardiac monolayers (10, 40). In addition, varying directions of propagation due to alternating active pacemaker centers hamper the analysis and interpretation of these parameters. Moreover, it is not entirely known how these biological variations affect intrinsic features of locally registered FPs at single electrodes of the MEA. When MEA technology, used to register FPs under hypothermic conditions, is combined with our in-house developed algorithm for pacemaker detection, analysis of local and global intrinsic FP parameters in a monolayer with multiple pacemaker centers is possible for each separate pacemaker center. Utilizing this approach we are able to demonstrate a relative change of up to 14% for CV and intrinsic FP parameters (FP_{rise}, FP_{MIN}, and FP_{pre}) caused by alternating propagation directions at different temperatures and individual electrodes of the MEA regardless of the used electrode configurations (Table 1).

Pacemaker centers. Our experimental data have demonstrated a frequent occurrence of endogenous multiple pacemaker centers in quasi-isotropic grown cell cultures at stable temperatures. Based on this observation we investigated the impact of alternating excitation directions on intrinsic FP features and identified significant differences. However, different beating rates lead to electrophysiological changes such as alteration of AP duration or upstroke velocity (29, 42, 51). To exclude beating-rate-induced effects, an analysis of the changes of the beating rate among competitive endogenous active pacemakers was performed showing, interestingly, almost similar frequencies. We conclude that an autonomous random changeover procedure between dominant and interfering pacemakers at stable temperature levels leads to a change in excitation direction at almost stable beating rates. Therefore, the observed alterations of intrinsic FP features are caused by excitation direction changes so that electrophysiological effects of different beating rates can be neglected. In addition to competitive pacemaker centers at stable temperatures, endogenous pacemakers respond differently to a temperature reduction by changing their spatial activation patterns. This also leads to a change of direction in dominant wave-front-propagation patterns. The question is: when does a pacemaker achieve the activation threshold to synchronize the surrounding tissue? Thereby two main factors play a crucial role (24):

1) The coupling conductance between the pacemaker center and surrounding cell structure.

2) The spatial extension of the pacemaker center (number of cells) and their intercellular coupling (morphology).

Hypothermia induces a decreased spontaneous depolarization of pacemaker cells, which results in a lower frequency of the pacemaker center (19, 35, 53). If during cooling another endogenous center exhibits a higher rate than the currently dominant center, this center becomes dominant.
In our case of randomly grown cell layers (see Fig. 1), it is not obvious how many potential pacemaker centers are inherent in the cell culture, how distinct their electrical coupling is, and how sensitive the beating rate of each center is when responding to hypothermal conditions. Additionally, the fact that in most cases the pacemaker regions are outside the MEA registration area hampered us to study the physiological mechanisms of hypothermia-induced pacemaker activation. Because

![Fig. 8. Temporal activation patterns of active pacemaker centers during cooling in the temperature range of 37 to 29°C. A: id 1 and id 3 are biological pacemaker centers while id 2 indicates an artificial stimulus. In the temperature range from 37 to 35°C the dominant pacemaker center 1 interferes with center 3. A change to dominant center 3 can be observed between 35 and 33°C. F: id 1 and id 3 to 5 are biologically autonomous pacemaker centers while id 2 indicates an artificial stimulus. Three temperature-induced changes of the dominant pacemaker center are observed. In both diagrams A and F, id 0 indicates wavefront propagation centers which could not be assigned by the detection algorithm. B and G: spatial distribution of pacemakers in the myocardial cell layer of the corresponding experiment (A and F). The square marks the MEA registration area. C, D, and E: isochronal maps calculated in the MEA registration area for all 3 active pacemakers corresponding to the experiment in A at 35°C. The numbers on the isochronal lines denote the wavefront propagation in milliseconds. The MEA covers an area of 1.4 × 1.4 mm.

![Fig. 9. A–E: selected boxplots of spread-dependent FP features for two different autonomous pacemaker centers in a single cell layer at 31 and 39°C. It is obvious that measured values of each feature, except for FPdur, differ as a function of location of the pacemaker center and temperature.](http://ajpheart.physiology.org/)

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00097.2014 • www.ajpheart.org
of the complexity of such highly random mechanisms and the lack of measuring pacemaker signals directly, we are not able to establish a predictive model on our experimental results for estimating the temperature-dependent alteration of pacemaker centers in time and space.

Conduction velocity. CV changes significantly with different orientations of myocardial cells as well as varying conductances and distributions of gap junctions. With the proposed method, relying on a spatially constant electrode position to calculate the velocity, the same cell layer segments can be measured repeatedly. This allows for a spatial and temporal comparison of the velocity in the experimental data. We could confirm a spread-direction dependency in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions. In particular, the effect of temperature on CV at single measurement points inside the culture for the examined temperatures during cooling. This direction-dependent effect can be explained by local matrix inhomogeneities of the cell layer.

*FP* <sub>rise</sub>. As shown, the detection of *FP* <sub>rise</sub> at single points in a quasi-isotropic myocardial cell layer is highly sensitive to the direction of propagation. Due to the linear relationship between *FP* <sub>rise</sub> and AP rise time, the observed changes indicate a possible direction-dependent alteration in the upstroke velocity of AP.

*FP<sub>MIN</sub>* and *FP<sub>pre</sub>*. The two prominent peaks *FP<sub>MIN</sub>* and *FP<sub>pre</sub>* in a FP are caused by the excitation of the local and surrounding tissue of the observed electrode, respectively, where *FP<sub>MIN</sub>* precedes in time with the maximum upstroke velocity of the AP and correlates to the Na<sup>+</sup> current (17). Our experimental data showed that the amplitudes of these parameters also exhibit direction-dependent characteristics at single points inside the cell layer.

*FP<sub>dur</sub>*. *FP<sub>dur</sub>* is an important indicator for the repolarization of myocardial tissue. It is known that hypothermia induces a prolongation of repolarization time that is coupled with a prolongation of the AP (1, 26). This effect is also evident in our experimental data. However, for the investigation of hypothermal-induced changes of FPs, it is important to know whether *FP<sub>dur</sub>* varies with different propagation directions. Statistical testing, taking into account *FP<sub>dur</sub>* for single electrodes, revealed in ~60% of experiments no significant difference between *FP<sub>dur</sub>* of the single pacemaker centers. However, because of the small magnitudes of the repolarization wave (*FP<sub>MAX</sub>*), the detection accuracy for local *FP<sub>dur</sub>* was limited.

**Conclusion.** In this study we demonstrated that alternating excitation directions has a significant impact on intrinsic electrophysiological parameters of registered FPs in randomly grown multicellular preparations of cardiac myocytes under normal and hypothermal conditions. In particular, the effect of spread-direction dependency could be experimentally verified and validated with established direction-dependent features such as CV or *FP* <sub>rise</sub> using MEA technology. In addition, we observed a spread dependency of FP spike amplitudes (*FP<sub>MIN</sub>* and *FP<sub>pre</sub>*). Furthermore, the observation of spread dependency at a stable temperature implies a similar effect also at altered...
temperature levels. This is essential in the analysis of hypothermal alterations since we demonstrated that cooling down a quasi-isotropic myocardial cell culture, and the fact that myocytes are highly sensitive to temperature changes (11), result in a spatial change of the dominant pacemaker center in a quasi-isotropic cell layer in almost all experiments. This also leads to a change of propagation direction as demonstrated. Bypassing this spread-dependent effect by application of artificial stimuli, however, has two main disadvantages: first, lack of beating rate information of the biological pacemakers, and secondly, the risk of possible alterations of electrophysiological parameters by artificial stimuli. Therefore, we can conclude that changes in propagation direction coming from alternating endogenous biological pacemakers need to be considered in the analysis and interpretation of electrophysiological mechanisms of non-patterned, randomly grown cell preparations of isolated cardiomyocytes at different temperature levels. Disregarding these observations can lead to misinterpretations and erroneous conclusions in the verification of research hypotheses in molecular and cellular electrophysiology.

APPENDIX

Pacemaker detection algorithm.

To distinguish registered wavefronts of different propagation direction, the algorithm computes the spatiotemporal distribution of all active pacemaker centers in and outside of the MEA registration area using a two-step approach (25):

1) Calculating the starting point of excitation for each individual registered wavefront based on the negative two-dimensional spatial gradient of the wavefront’s arrival time within the area of the electrode array.

2) Identifying local clusters of active pacemaker centers in the cell culture using the information of the first step. Applying this paradigm assigns each individual registered wavefront to an active pacemaker center. This information permits a direction-dependent separation of propagation features for further analysis. Figure A1 shows superimposed isochronal maps of all single beats (n = 949) of a selected cell layer separated by the differentiated excitation directions.

Distance-dependent behavior of FP rise.

To confirm the distance-dependent behavior of FP rise observed by Halbach et al. (17), a correlation analysis was performed. For each temperature level and each pacemaker center, the Pearson correlation coefficient (r value) was calculated where the distance between pacemaker and electrodes was correlated with the median value of FP rise for each single electrode. The box plots in Fig. A2 show the calculated r values for the different temperatures. It is clear that we were not able to confirm a significant relationship between FP rise and the distance to the pacemaker centers (Fig. A2A). The same analysis was performed solely for the artificial stimuli (without autonomous pacemaker centers), showing a similar result, however, with a slight negative correlation coefficient of about -0.2 (Fig. A2B).

Interestingly, Spach et al. (48) also found a correlation between AP upstroke and the time interval between FP pre and FP min. This observation could be confirmed indirectly by an FP rise increase with decreasing temperatures \[r = 0.87 (SD 0.25), P < 0.01\]. Therefore, the time interval [FP pre, FP min] is also expected to increase analogously to FP rise and is a suitable measure that can be used alternatively to FP rise for the investigation of temperature induced changes of the myocardial AP. A further advantage is, if signals show strong baseline drifts, that the FP pre - FP min interval compared with FP rise is almost independent of baseline variations.

Impact of artificial stimuli on CV and intrinsic FP parameters.

It is known that rapid electrical stimulation (RES) causes alterations in myocardial gap junctions (modulated by the expression of the gene Connexin 43), which resulted in an increase of CV after ~60 min (23, 39, 40). In contrast to the reported stimulation frequency of RES (~3 Hz) our stimulation frequency was in the range of 0.7 to 2 Hz with a maximal duration of 30 s per stimulation sequence. However, visual inspection of the data reveals a remarkably strong variation of intrinsic FP parameters, caused by biological and artificial stimuli (for example, see FP rise...
in Fig. 2, B and C). To investigate the influence of the applied stimulus, we hypothesized as follows.

An artificial stimulus has a stronger impact on the FP morphology and CV than biologically autonomous pacemakers, and therefore, the relative median changes of these parameters between biological pacemakers and artificial stimulus are significantly different than those found solely among the natural pacemakers.

To prove this hypothesis we selected only those data from our measurements where, at a stable temperature, at least two biological pacemakers and the artificial stimulus were active. Based on these data we repeated previously described analyses for all intrinsic FP features. In a first step, the artificial stimuli were excluded, considering only biological pacemakers. In the second step, the artificial stimulus was included to compare the common effects of biological and artificial stimuli. The results revealed a slight, but significant, difference (p < 0.01, Mann-Whitney U-test) for all investigated features (Fig. A3). In detail, FP_rise and FP_MIN increased in the presence of an artificial stimulus, where CV, FP_pre, and FP_dur decreased compared with the natural pacemakers. Therefore we could confirm that artificial stimuli have a stronger impact on the FP morphology and CV. Interestingly, compared with the aforementioned, insignificant relationship between repolarization time (FP_min) and direction dependency of different biological pacemakers (see FP_dur in RESULTS), we found a significant difference between biological and artificial stimuli (p < 0.01) (Fig. A4). To conclude, the impact is small, but significant, in all investigated FP features and CV. However, to validate these observations, in particular if these changes are caused by electrophysiological variations between biological and artificial stimuli or merely due to an increase in beating rate, additional comprehensive experiments are needed.

Links to raw data and movies.

Selected raw data in MATLAB format (The Mathworks, Natick, MA) and movies, illustrating changes in excitation direction are available at: http://www.umiit.at/page.cfm?path=departments/technik/iebe/tools/propagation_direction_dependent_fps&switchLocale=en_US

AUTHOR CONTRIBUTIONS

Author contributions: R.K. and C.B. conception and design of research; R.K. and M.S. performed experiments; R.K., M.H., and F.H. analyzed data; R.K. interpreted results of experiments; R.K. prepared figures; R.K. and M.S. drafted manuscript; R.K., M.S., M.H., F.H., and C.B. edited and revised manuscript; R.K., M.S., M.H., F.H., and C.B. approved final version of manuscript.

REFERENCES


