Ventricular Slices of Adult Mouse Hearts - a new Multicellular *In Vitro* Model for Electrophysiological Studies

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Key Words

Myocytes • Cardiac/*physiology • Electrophysiology/*methods • Action potentials • Cardiac field potentials • Microelectrode array • Heart/*physiology • Animal models • Slices

Abstract

Aim: We established a preparation of adult murine ventricular slices suitable for electrophysiological recordings as a new *in vitro* model of adult myocardium with preserved *in vivo* tissue structure. **Methods:** Short axis slices (thickness: 150 μ m) of adult murine ventricles were prepared with a microtome. Sharp glass electrodes were used for measurements of action potentials (APs) at stimulation frequencies of 2 Hz and 10 Hz. Field potential (FP) recordings by means of microelectrode arrays (MEAs) were performed to map excitation spread. **Results:** APs showed the characteristics of adult murine ventricular APs: (i) a stable resting membrane potential, (ii) a fast upstroke and (iii) a fast phase 1 repolarization. Application of the Na⁺ channel blocker lidocaine (30 μ M) led to a decline of upstroke slope, amplitude and conduction speed.

The unspecific K⁺ channel blocker 4-aminopyridine (5 mM) caused a prolongation of APD₅₀. The excitation spread was homogenous throughout the ventricular wall. **Conclusion:** Adult murine ventricular slices are electrophysiologically intact and respond physiologically to cardioactive drugs. Thus, they provide a new multicellular *in vitro* model of adult cardiac tissue suitable for electrophysiological investigations, which in future could be used to study the functional integration of stem cells transplanted in infarcted hearts *in vivo*.

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Introduction

Heart function critically depends on an intact structure on the tissue level, characterized by cardiomyocyte size and orientation, gap junction distribution and properties of non-myogenic cells as well as extracellular matrix. Multicellular *in vitro* models used to study electrophysiology of cardiac tissue mostly do not possess a preserved *in vivo* structure (e.g. monolayers of dissociated cardiomyocytes [1-3], embryoid bodies [4]). Therefore findings on speed and path of conduction as

well as on arrhythmias must be interpreted carefully and cannot be transferred to the *in vivo* situation without major limitations.

Moreover *in vitro* studies of functional integration of stem cell-derived cardiomyocytes into cardiac tissue, which have recently been conducted [1-3] and are crucial for a safe and efficient cell replacement therapy of myocardial infarction, underlie the same limitations. To overcome these hindrances, a preparation with preserved *in vivo* structure and normal electrophysiological and pharmacological properties is needed.

These demands could be fulfilled by arterially perfused ventricular wedge preparations [5]. However, this kind of preparation leads to three-dimensional tissue blocks not suitable for two-dimensional mapping of excitation spread. The latter restriction holds also true for the usage of superfused whole ventricular walls [6, 7], which allow direct access to subepicardial and subendocardial but not to intramural layers.

An alternative approach is the preparation of thin slices of ventricular tissue. Tissue slices are well established in neurophysiological research and led to a considerable improvement of our understanding of integral neuronal function. In 1990, Burnashev et al. [8] described a method to obtain slices of neonatal rat hearts allowing to perform patch clamp recordings of single cardiomyocytes within the slices. However, resting membrane potentials were in an unphysiological range of -30 to -65 mV. Since then, heart slices have not been used for electrophysiological investigations up to the establishment of murine embryonic heart slices by our group in 2005 [9]. This method provides viable and electrophysiologically intact thin slices (thickness: $300\text{ }\mu\text{m}$), which are suitable for high-resolution investigations of two-dimensional excitation spread as well as arrhythmias. However, the characteristic structure of adult myocardium (e.g. cell size and gap junction distribution) as well as its typical electrophysiological properties, cannot be simulated sufficiently by means of immature cardiac tissue [10].

Modifying the method used to obtain embryonic ventricular slices [9], we established a new preparation of viable and electrophysiologically as well as pharmacologically intact slices of murine adult ventricles providing a preserved *in vivo* structure. This preparation will be useful for studying the effects of cardioactive drugs and the pathophysiological impact of genetical modifications on two-dimensional excitation spread and arrhythmias in adult myocardium. Moreover ventricular slices of adult mouse hearts provide a unique basis for

Fig. 1. Picture of a short axis slice of a murine adult ventricle (thickness: $150\text{ }\mu\text{m}$).

future studies of the functional integration of cells transplanted *in vivo* to repopulate injured myocardium.

Materials and Methods

Preparation of slices

All experiments conformed to the guidelines of the local animal welfare committee. 15 min after intraperitoneal application of $100,000\text{ IU/kg}$ heparin sodium (Hoffmann-La Roche, Grenzach-Wyhlen, Germany), adult mice (>5 weeks old) of the strain HIM:OF 1 were killed by cervical dislocation. Hearts including the great vessels were removed quickly and the aorta was perfused retrogradely via a syringe with oxygenated ice-cold Ca^{2+} -free Tyrode's solution (composition in mmol/l: NaCl 136, KCl 5.4, NaH_2PO_4 0.33, MgCl_2 1, glucose 10, hepes 5, 2,3-butanedione monoxime 30; pH 7.4 adjusted with NaOH). Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany). Ventricles were dissected from the atria, valves and great vessels and embedded in 4% low-melt agarose (Roth, Karlsruhe, Germany). Agarose-embedded ventricles were glued on a specimen holder by means of cyanoacrylate glue (Pattex/Henkel, Duesseldorf, Germany). Short axis slices (thickness: $150\text{ }\mu\text{m}$; see Fig. 1) were cut using a microtome (DTK-2000, Dosaka, Kyoto, Japan) with steel blades (Campden Instruments, Leicester, England) and stored in the standard ice-cold Tyrode's solution, which was supplemented with 0.9 mmol/l Ca_2Cl . After 30 min, slices were transferred into DMEM without serum (Gibco/Invitrogen). Before measurements were carried out, slices were kept in DMEM for at least 30 min.

Fig. 2. APs recorded under control conditions. Electrical stimulation was performed at 2 Hz (left panel) and 10 Hz (right panel), respectively. All measurements revealed a stable resting membrane potential, a very fast upstroke and a fast phase 1 repolarization. APs at 2 Hz had a pronounced plateau leading to a prolonged APD₉₀, which was diminished at 10 Hz. Stimulation frequencies up to 10 Hz led to a stable and long-lasting 1:1 generation of action potentials.

AP recordings

Intracellular recordings of APs were performed with sharp electrodes (20–50 M Ω when filled with 3 mol/l KCl) made of borosilicate glass capillaries (WPI, Sarasota, USA). Signals were amplified with a SEC-10LX amplifier (npi electronic, Tamm, Germany) and acquired with the Pulse software (HEKA, Lambrecht/Pfalz, Germany). Data were analyzed offline with the Mini Analysis program (Synaptosoft, Fort Lee, USA). All measurements were performed at 37°C in DMEM (concentrations of inorganic salts in mmol/l: CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.3, NaHCO₃ 44, NaCl 110, NaH₂PO₄ 0.9) without serum bubbled with 5% CO₂ and 95% O₂. For investigations of pharmacological reactions, 30 μ mol/l lidocaine (B. Braun, Melsungen, Germany), 5 mmol/l 4-aminopyridine or 30 μ mol/l verapamil were added to the DMEM.

A standardized beating frequency of either 2 Hz or 10 Hz was applied using a unipolar stimulation electrode described elsewhere [11]. The focal unipolar stimulation allowed an estimation of relative changes of conduction speed by evaluating the latency between stimulation artifact and onset of the AP upstroke (see Fig. 3). Since the distance between stimulation and recording electrode (generally > 1 mm) as well as the path of excitation spread could not be determined exactly, only relative changes and no absolute values of conduction speed were evaluated. Relative conduction speed was calculated as follows: The reciprocal of the absolute latency between stimulation artifact and onset of the AP upstroke at any time was divided by the reciprocal of the absolute latency under control conditions.

FP recordings with MEAs

For synchronous multifocal FP measurements, slices were placed on MEAs (Multi Channel Systems, Reutlingen,

Germany) with an inter-electrode distance of 200 μ m and perfused with DMEM without serum bubbled with 5% CO₂ and 95% O₂ at a temperature of 37°C. Activation maps were generated by means of a custom-made program based on MATLAB (The Mathworks, Natick, USA) using the time points of maximal downstroke velocity determined for each electrode as described [9, 12]. With the gradient function of MATLAB, vectors reflecting the direction of excitation spread and the magnitude of the delay of activation were calculated from the matrix of activation time points.

Statistics

AP parameters recorded under control conditions at 2 Hz and 10 Hz were tested for statistical significance by unpaired Student's *t*-test or, if normality test failed, by Mann-Whitney rank sum test. All data are presented as mean $\pm$ S.E.M. *n*-values refer to the number of measurements, not to the number of slices.

Results

Viability of the slices

Shortly after washout of 2,3-butanedione monoxime, focal stimulation led to a circular transmural beating of the slices, which involved both ventricles and the inter-ventricular septum. The slices generally could be stimulated with frequencies up to 10–12 Hz. Even at these high stimulation frequencies, a stable 1:1 generation of APs and contractions existed (see Fig. 2). Stable electrophysiological properties and circular beating were

Fig. 3. Effect of Na⁺ channel blockage. Application of 30 μmol/l lidocaine led to a reduction of $V_{10-90\%}$ (A), amplitude (B) and relative conduction speed (C). Relative conduction speed was calculated from the latency between stimulation artifact and onset of the AP (for details see Materials and Methods), which increased after administration of lidocaine (C+D). APs instead of stimuli have been superimposed in (D) to demonstrate reduction of upstroke velocity and amplitude due to the effect of lidocaine. All effects were reversible after washout.

maintained for at least 4 hours.

Without stimulation, most slices showed spontaneous contractions after washout of 2,3-butanedione monoxime. These spontaneous contractions were reduced with increasing time in culture. Although many AP measurements have been performed, no pacemaker-like APs were recorded.

AP recordings

APs recorded under control conditions showed uniform morphologies and parameters typical for murine adult APs, predominantly characterized by a fast phase 1 repolarization (see Fig. 2). Measurements at a stimulation frequency of 2 Hz (n=30) revealed a stable resting

membrane potential of -78.2 ± 0.8 mV, a very fast upstroke with a 10-90% upstroke velocity ($V_{10-90\%}$) of 133.0 ± 3.6 V/s, an amplitude of 91.1 ± 1.1 mV and the characteristic fast phase 1 repolarization with an APD₅₀ of 3.4 ± 0.2 ms (see Table 1). APD₉₀ measured at 2 Hz (86.5 ± 1.6 ms) was significantly prolonged in comparison to previously reported values (67.4 ± 2.7 ms (at 5 Hz) [13]), but could be reduced by simulating the physiological murine heart rate [14] by a stimulation frequency of 10 Hz (APD₉₀ at 10 Hz: 43.6 ± 3.3 ms). Stimulating the slices with a frequency of 10 Hz led to a significant reduction of the amplitude to 80.9 ± 3.4 mV and a significant decrease of $V_{10-90\%}$ to 92.1 ± 11.9 V/s.

Voltage-dependent Na⁺ and K⁺ currents

Stimulation frequency (Hz)	Resting membrane potential (mV)	Amplitude (mV)	V _{10-90%} (V/s)	T _{10-90%} (ms)	APD ₅₀ (ms)	APD ₉₀ (ms)	n
2	-78.2±0.8	91.1±1.1	133.0±3.6	0.56±0.01	3.4±0.2	86.5±1.6	30
10	-76.3±1.4	80.9±3.4	92.1±11.9	0.87±0.28	4.6±1.0	43.6±3.3	12
	P = 0.213	P < 0.001	P < 0.001	P < 0.001	P = 0.237	P < 0.001	

Table 1. Statistical analysis of AP parameters. AP parameters (mean ± S.E.M.) determined under control conditions at a stimulation frequency of 2 Hz or 10 Hz. Amplitude, V_{10-90%}, T_{10-90%} and APD₉₀ differ significantly. V_{10-90%}: 10-90% upstroke velocity; T_{10-90%}: 10-90% upstroke duration.

Fig. 4. Effect of K⁺ channel blockade. Application of 5 mmol/l 4-aminopyridine evoked a prolongation of APD₅₀, which was reversible after washout. A: Time course of the APD₅₀. B: Representative APs under control conditions and at maximal effect of 4-aminopyridine.

predominantly influence murine adult ventricular APs. To study the effects of known potent Na⁺ and K⁺ channel blockers on APs of cardiomyocytes within the slices, we applied lidocaine, a blocker of voltage-dependent Na⁺ channels [3], as well as 4-aminopyridine, an unselective blocker of voltage-dependent K⁺ channels [15]. Application of 30 μmol/l lidocaine (see Fig. 3 for a representative example) evoked a decline of relative conduction speed, amplitude (78.8±2.0 mV at 30 μmol/l lidocaine; n=16; P<0.001 vs. amplitude under control conditions) and V_{10-90%} (86.1±4.9 V/s at 30 μmol/l lidocaine; n=16; P<0.001 vs. V_{10-90%} under control conditions). Addition of 5 mmol/l 4-aminopyridine (see Fig. 4 for a representative example) prolonged APD₅₀ (42.2±2.5 ms under 5 mmol/l 4-aminopyridine; n=9; P<0.001 vs. APD₅₀ under control conditions). Moreover

contractility of the slices was enhanced, according to previous reports [16]. All effects of lidocaine and 4-aminopyridine were reversible upon washout.

Plateau duration could be slightly reduced by Ca²⁺ channel blockade due to application of 30 μmol/l verapamil (data not shown). No obvious rundown of Ca²⁺ currents, i.e. no time-dependent reduction of plateau duration, was observed during any measurement, which lasted up to 80 min.

MEA measurements of excitation spread

Synchronous multifocal FP recordings by means of MEAs showed electrical activity at electrodes covered by subendocardial, intramural and subepicardial tissue. FP morphology was uniformly dominated by FP_{PRE} (i.e. the first positive deflection of the FP [11]) and FP_{MIN} (i.e.

Fig. 5. Synchronous multifocal FP recording. A: Schematic illustration of the position of the slice on the MEA. The recording electrodes covered subendocardial, intramural and subepicardial tissue. B: Map of the delay of time points of maximal downstroke velocity at each electrode. Excitation spread was homogenous throughout the recording area. The size of the vectors reflects the magnitude of the delay, the direction of the vectors shows the direction of excitation spread. C: Original FPs. The stimulation artifact precedes each FP.

Fig. 6. Decrease of FP_{DUR} at 10 Hz as compared to 2 Hz. According to the shortened APD₉₀ (see Fig. 2), FP_{DUR} is considerably shorter at 10 Hz than at 2 Hz. Each FP is preceded by the stimulation artifact.

the first negative deflection of the FP [11]). Mapping of the delay of time points of maximal downstroke velocity at each electrode revealed a homogenous spread of excitation (see Fig. 5).

FP parameters are known to correlate to AP parameters, e.g. FP duration (FP_{DUR} : duration between FP_{MIN} and the last FP maximum) correlates to APD_{90} [11]. Corresponding to the decrease of APD_{90} at 10 Hz as compared to 2 Hz, FP_{DUR} at 10 Hz was considerably shorter than at 2 Hz (see Fig. 6).

Discussion

Presently, most studies on cardiac electrophysiology of multicellular preparations are based on immature cardiomyocytes, e.g. neonatal rat cardiomyocytes [2], and thus do not resemble the *in vivo* structure of adult cardiac tissue regarding cell size, gap junction distribution, arrangement of non-myogenic cells and extracellular matrix. Results concerning excitation spread, arrhythmias or functional integration of co-cultured stem cells [1-3] cannot be reliably transferred to the *in vivo* situation. To overcome the limitations of currently used models, we have established a novel method to prepare slices of murine adult ventricles, which meet all conditions necessary for an appropriate, advanced model of the adult heart: mature cardiomyocytes, multicellularity, preserved *in vivo* structure as well as intact electrophysiology and reactions to cardioactive drugs.

Focal stimulation of murine adult ventricular slices led to a circular contraction involving both ventricles as well as the interventricular septum. Stimulation frequencies of 10-12 Hz, which correspond to the physiological murine heart rate [14], were tolerated and evoked rhythmic APs and contractions pointing to a healthy state of the preparations. Spontaneous contractions observed in most of the unstimulated slices may be caused by cardiomyocytes sublethally damaged by the slicing procedure. Former histological evaluations in embryonic heart slices showed that mechanical damage due to the slicing procedure is inflicted to the cut surface of the slices [9]. However, although numerous AP measurements have been performed, only stable resting membrane potentials were recorded. Thus a potential sublethal cell damage is presumably limited to very few cells.

AP recordings under control conditions showed that cardiomyocytes within the slices were electrophysiologically intact. Besides a stable high resting membrane potential, all APs recorded had a fast upstroke,

a high amplitude and the short APD_{50} characteristic for murine adult ventricular APs. Mean values of these parameters correspond well to values reported in the literature [6, 7, 14]. The prolonged APD_{90} measured at 2 Hz could be reduced to reference values [14, 17, 18] by an increase of the stimulation frequency to 10 Hz. As it was not possible to clamp voltage in the slices, we could not determine whether the prolongation at 2 Hz was influenced by an increase of depolarizing Ca^{2+} current or an incomplete activation of repolarizing K^{+} currents. Since an activation of ATP-dependent K^{+} channels, which are activated under hypoxic conditions, would lead to a shorter APD_{90} [13], the prolongation at 2 Hz suggests that the slices are in a good metabolic state.

The significant reduction of the amplitude and $V_{10-90\%}$ at 10 Hz as compared to 2 Hz may be explained by the recovery from inactivation kinetics reported for cardiac voltage-dependent Na^{+} channels [19]. Since a 100 ms interval between successive APs is not sufficient for a complete recovery from inactivation, the fast Na^{+} current, which predominantly determines the amplitude as well as the fast upstroke velocity, is presumably reduced at 10 Hz.

The effects of lidocaine and 4-aminopyridine on AP parameters correspond well to known effects of these substances on single cardiomyocytes or aggregates of dissociated cardiomyocytes [3, 20]. The reduction of conduction speed, a typical effect of lidocaine, was estimated by calculating the increase of the latency between stimulation artifact and onset of the AP upstroke. Relative changes of conduction speed after application of lidocaine were thus proven. Since the exact path of excitation spread between stimulation and recording electrode is unknown, values of absolute conduction speed could not be determined.

Using synchronous multifocal FP recordings with MEAs to demonstrate transmural electrical activity and prove homogenous excitation spread, we found, in line with visible transmural beating, electrical activity in subepicardial, intramural and subendocardial regions. This ubiquitously distributed electrical activity corresponds well to previous results from murine embryonic ventricular slices [9]. FP_{DUR} , in parallel to APD_{90} , was reduced at 10 Hz as compared to 2 Hz. The correlation of FP_{DUR} and APD_{90} has not yet been described for adult cardiac tissue, but matches with previous findings from embryonic cardiomyocyte monolayers [11].

In conclusion, acute slices of murine adult ventricles are viable, electrophysiologically intact and react normally to cardioactive drugs. Due to the preserved *in vivo*

structure, they may, for future studies, provide unique means for investigating infarcted myocardium and the functional integration of stem cells transplanted *in vivo*. Furthermore, they are an ideal model of adult myocardium to study the impact of pharmacological substances or genetical modifications on two-dimensional excitation spread and arrhythmias.

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