Establishment and Characterization of a Mouse Embryonic Heart Slice Preparation

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Key Words

Myocytes, cardiac/*physiology • Action potentials • Electrophysiology/*methods • Electrophysiology/*embryology • Heart/*physiology • Animal models

preserved. Conclusion: Functionally intact heart slices can be generated from murine embryos.

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Abstract

Background: In contrast to isolated cells, the anatomic and functional integrity of tissue slices remains preserved. **Aim of the study** was to establish the slice technique in embryonic mouse hearts in order to perform physiological and pharmacological investigations of wild-type mice and genetically engineered mouse models of heart disease. **Methods:** Ventricular slices (thickness: 300 µm) were cut from agar-embedded embryonic mouse hearts (ED 16.5–18.5) with a vibratome. Histology, immunostaining with markers for apoptosis induction, intracellular recordings with sharp electrodes and field potential recordings using microelectrode arrays were performed to assess viability. **Results:** Slices exhibited normal histology without prominent signs of apoptosis for at least 24 hours. Intracellular recordings revealed the typical electrophysiological fingerprint of ventricular cardiomyocytes. Field potential recordings proved that adrenergic and muscarinic signaling was

Introduction

The advent of the brain slice preparation led to a dramatic improvement of the understanding of cerebral activity because it has enabled studies of cellular function in the context of an intact tissue [1]. This is particularly important for structures like the brain and the heart in which intercellular cross-talk is critical for complex organ function. Furthermore, aggressive dissociation methods required to prepare isolated cells, often have adverse effects on the function of ion channels and receptors. Thus, we sought to establish an intact heart slice preparation so that we might better understand mechanisms of cardiac physiology in the future. To date, relatively little progress has been made in establishing a viable heart slice preparation [2, 3] and in recording physiological activity of cardiac slices [4]. This is in part due to the fact that the heart contains a significant amount

of extracellular matrix and is expected to exhibit hypercontraction in response to even small increases in extracellular K^+ , which leaks out of damaged cells. Burnashev et al. [4] reported the first patch-clamp recordings using neonatal rat heart slices. Although they provided no information on the structural integrity or viability of the tissue, they did report that a significant number of cells had depolarized resting potentials. Consequently, for many years electrophysiological studies of cardiac function have relied primarily on preparations of isolated cardiomyocytes. Because of the increasing importance of transgenic and gene-targeted mice being developed to study cardiac electrophysiopathology [5] we developed this model in murine heart tissue.

Materials and Methods

Harvesting of tissue and preparation of slices

Experiments were carried out according to the guidelines of the local animal welfare committee. Mice were superovulated, time-mated and tissue harvested between days 16.5-18.5 post coitum [6]. Isolated hearts and slices were kept in ice-cold (4°C) Ca^{2+} -free Tyrode's solution (see [3] for composition), continuously gassed with pure oxygen; CaCl_2 was added as indicated. All substances were purchased from Sigma Chemical Co. unless otherwise stated. 4% low-melting agarose (Roth, Karlsruhe, Germany) dissolved at 70°C and maintained liquid at 34°C was poured over the hearts, rapidly chilled and was allowed to solidify on ice. Agarose-embedded hearts were fixed onto a specimen holder using cyanoacrylate glue (Pattex, Henkel, Germany). Ventricles were sectioned into 300- μm -thick slices along the short axis with a microtome (Leica VT1000S, Leica Microsystems, Germany) using steel blades (Campden Instruments, Leicester, England).

Histology

For histological and immunohistochemical examination, 7 μm thick cryosections were prepared from the slices. Immunohistochemistry was performed as described [7] using rabbit anti-active-Caspase-3 antibody (1:500, Pharmingen, San Diego, Ca, USA) and rabbit anti-poly(ADP-ribose) polymerase (PARP) p85 fragment antibody (1:250, Promega, Madison, WI, USA) as primary antibodies.

Action potential (AP) and field potential (FP) recordings

AP recordings were performed with sharp glass micropipettes and FP recordings with blunt glass micropipettes in an interface-type chamber superfused with Tyrode's solution (Ca^{2+} 0.9 mM; 32°C) (see also [8]). Whole-cell current clamp recordings of isolated ventricular cardiomyocytes were obtained as reported [9] using external Tyrode's solution containing Na^+ 140 mM and Ca^{2+} 3.6 mM.

Microelectrode arrays

For microelectrode array (MEA) recordings, slices were transferred to ice-cold Tyrode's solution (0.9 mM Ca^{2+}) bubbled with 100% oxygen for 30 minutes, plated on planar MEAs (Multi Channel Systems, Reutlingen, Germany) using cooled Iscove's medium containing 20% heat inactivated fetal calf serum (Gibco Inc., Germany) and equilibrated with 5% CO_2 at 37°C [10]. One hour before electrical recordings were obtained, Iscove's medium was replaced with Tyrode's solution (Ca^{2+} 1.8 mM). Spontaneous or electrically stimulated field potentials were recorded using a MEA60 system and analyzed off-line [10]. A bipolar external electrode (diameter: 40 μm) applying a square pulse (5-10 V; 5 ms) was used for stimulation. The timing of maximal negative intrinsic deflection ($dV/dt_{\min}$) of the recordings was calculated and averaged for each electrode to generate color-coded activation maps [11].

Statistics

All data are presented as means $\pm$ standard deviations (SDs). Heart rate analysis was performed using paired t-tests with Bonferroni adjustment for multiple comparisons. One-way analysis of variance (ANOVA) and Tukey's post-test analysis were used for comparison of AP characteristics after equal variances were determined by the Levene procedure. Statistics were calculated using SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA). P values <0.05 were considered significant.

Results

Histology

Hematoxylin and Eosin (H & E) (Fig. 1A) and trypan blue staining (Fig. 1B) was performed to evaluate the structure of cardiac tissue slices. This analysis demonstrated that the histological structure was well-preserved, although some disruption of tissue integrity with trypan blue positive cardiomyocytes was observed in two to three of the most superficial cell layers (approx. 40 μm). We assessed the degree of apoptosis in these cells by staining for active-Caspase-3 (Fig. 1C) and/or PARP p85 fragment (Fig. 1D). This analysis revealed that active-Caspase-3 and PARP p85 fragment staining localized to trypan blue positive areas. Thus, the cells throughout the cardiac slice except for those in the surface layers were viable and intact for the first 24 hours after preparation (Fig. 1 C, D).

AP and FP recordings

Intracellular AP and FP recordings were obtained primarily from slices without spontaneous beating; however, qualitatively useful recordings were also acquired from contracting slices. Intracellular recording

Fig. 1. Histology: A) H & E staining revealed viable myocardial tissue. B) The vital staining with Trypan Blue labeled damaged cells within the 2-3 surface layers of the slice. C, D) Staining for aCaspase-3 (C) or 85 kDa PARP (D) indicated apoptosis of cardiomyocytes on both sides of the slice, whereas no immunostaining was seen in the remaining myocardium. Bar A = 100 μ m; B = 60 μ m; C-D = 40 μ m, arrows indicate positively stained cells.

Fig. 2. Intracellular recordings from heart slices: A) FP (upper trace) and AP (lower trace) recordings from a heart slice that displayed both spontaneous (gray; n=6) and triggered (black; n=7) APs. Comparison of stimulated APs (RMP -64 ± 3 mV, dV/dt 1.19 ± 0.10 V/s, AMP 86 ± 3 mV, APD50 134 ± 2 ms, APD90 193 ± 8 ms; n=3) and spontaneous APs (RMP -64 ± 1 mV, dV/dt 0.73 ± 0.02 V/s, AMP 95 ± 1 mV, APD50 127 ± 5 ms, APD90 190 ± 17 ms; n=5) showed only significant differences in dV/dt. B) Typical spontaneous AP of an isolated ventricular cardiomyocyte. C) FPs (upper trace) and APs (n=15) (lower trace) triggered by paired stimuli showed refractory periods of 230-250 ms.

Fig. 3. Pharmacological testing: A) Addition of KCl (50 μ l, 2 M) to the bath resulted in a strong depolarization of the RMP. Continuous perfusion of the recording chamber with normal solution led to a gradual recovery of the RMP (arrow; interval between traces: 10 s). The first two APs after recovery displayed prolonged APDs because of an extended repolarization phase (arrow heads). B) Lengthening of the repolarization phase by linopirdine (10 μ M).

Fig. 4. Spreading of electrical excitation: A) MEA recordings of local FPs averaged over 400 events. B) Two-dimensional color-coded map illustrating the delay of dV/dt_{min}. Isochronic lines suggest impulse propagation in a clockwise direction. Pink areas mark the position of the slice, black dots indicate MEA electrodes.

Table 1. Characteristics of stimulated APs recorded from myocardial slices and spontaneous APs of isolated ventricular cells. Values are means $\pm$ SD. N.s., non-significant ($P>0.05$).

Parameter	Slice (stimulated; n=8)	Isolated Cell (spontaneous; n=27)	P-value
RMP (mV)	-65 $\pm$ 8	-65 $\pm$ 5	n.s.
AMP (mV)	82 $\pm$ 14	101 $\pm$ 9	<0.001
APD50 (ms)	116 $\pm$ 18	74 $\pm$ 25	<0.001
APD90 (ms)	191 $\pm$ 25	129 $\pm$ 35	<0.001

from single cells within slices could be maintained for up to 20-45 minutes. APs exhibited a fast overshooting upstroke and a broad plateau phase typical of late stage embryonic ventricular cardiomyocytes (Fig. 2A, lower panel). APs of a cell within a slice that exhibited both stimulated and spontaneous APs were of similar shape (Fig. 2A, lower panel). FPs consisted of a sharp biphasic transient, which lasted approximately 20 ms and corresponded to the fast upstroke of the intracellular recording. A prolonged low voltage deflection usually ending with a negative field coincided with the plateau and repolarization phase of the AP (Fig. 2 A).

APs recorded in slices were compared to current clamp recordings of isolated ventricular cardiomyocytes from embryos of the same differentiation stage. The measurements yielded no significant differences in the resting membrane potential (RMP). However, the amplitude of APs was smaller and the APD50 and APD90 were significantly longer in stimulated cardiomyocytes within the slice than in spontaneously beating isolated cells (Fig. 2B, Tab. 1). To demonstrate another biophysical characteristic, the refractory period of stimulated cardiomyocytes was assessed with a double pulse stimulation protocol. The duration (230-250 ms) in these experiments corresponded to the APD at 92% repolarization (Fig. 2C). Addition of 50 μ l 2 M KCl solution (n=2 slices) resulted in an immediate massive depolarization of the RMP (Fig. 3A). Instead of producing a hypercontraction, the membrane potential recovered to normal values over a period of 90-120 seconds upon continued perfusion with normal solution. During recovery, the first APs that could again be triggered were prolonged; thereafter APs became identical to those before K⁺ administration (Fig. 3A).

In order to assess the utility of this model for pharmacological studies, linopirdine, a specific KCNQ channel blocker, was administered. Linopirdine (10 μ M) induced a delayed repolarization as indicated by the prolongation of the APD50 by 15 ms that could almost be reversed shortly after washout (Fig. 3B). Thus, these results demonstrate that this cardiac slice preparation

recapitulates responses previously observed for single cardiomyocytes.

MEAs

MEA recordings of FPs were performed on slices exhibiting spontaneous contractions. Spontaneous beating, in most cases throughout the whole slice, was observed in nearly half of the slices after the temperature was increased to 37°C and Ca²⁺ was added to the extracellular solution. The proportion of spontaneously beating slices remained constant for at least 60 hrs after slicing (median =43%, range =25-48%; n=9) and a few slices even maintained spontaneous rhythmic contractions for one week. The average beating frequency of contracting slices was 56 $\pm$ 38 bpm (n=36).

An example illustrating the feasibility of measuring the spread of electrical excitation in a spontaneously beating slice is shown in Fig. 4B and is based on the timing of the maximal negative intrinsic deflection (dV/dt_{min}) of the FPs displayed in Fig. 4A. A delay map calculated from these results demonstrated a circular conduction route for the electrical impulse. A spontaneously beating slice could be paced using external electrical stimulation resulting in synchronous contractions of the whole slice.

We used pharmacological agonists of muscarinic and beta-adrenergic receptors to evaluate whether these receptors produced the expected modulation of the beating frequency (chronotropy). Isoprenaline (ISO, 50 nM) induced an immediate increase of beating rate reaching a median of 156% (range: 105-289%) of the baseline. This response attenuated 4 min after the beginning of the ISO application (median =134%, range =89-240%; n=8) (Fig. 5 A, C), which is consistent with desensitization of the beta-adrenoceptor. The muscarinic agonist carbachol (CCh, 10 μ M) significantly decreased the beating frequency to 26% of the control rate (range: 0-67%), and on average this suppression persisted for 4 minutes after CCh application (median =44%, range = 0-65%; n=8) (Fig. 5 B & D).

Fig. 5. Hormonal modulation of chronotropy: A, B) Original traces recorded with MEAs showing positive (A) and negative (B) chronotropy of spontaneously beating ventricular heart slices in response to ISO (50 nM, A) and CCh (10 μ M, B). The effect was almost completely reversed by washout. C, D) Beating rates during the first 4 min after ISO (n=8, C) or CCh (n=8, D) administration. Each line shows the beating rate of a single slice.

Discussion

Here we demonstrate for the first time that viable ventricular slices can be generated from murine embryonic hearts. Standard histological staining in combination with immunohistological analysis for apoptosis markers demonstrated that cardiac slices were structurally intact except for damage to the outer 2-3 cell surface layers. The viability of this preparation was further validated by the fact that at least 50% of slices exhibited spontaneous beating for up to one week after preparation and that the excitation spread throughout the whole slice. While spontaneous beating in adult cardiomyocytes indicates cell damage and Ca^{2+} overload, it is a typical property of healthy embryonic heart cells [9].

Intracellular recordings of cardiomyocytes in slices revealed a physiological membrane potential and a spike and dome AP morphology. These properties are comparable to those previously described in late embryonic cardiomyocytes by us and other groups [12]. Remarkably, in the slice preparation, the APD was longer than in isolated cardiomyocytes or endocardial right ventricular cells [12]. This as well as the extended refractory period might be due to elevations in extracellular K^+ within the slice where diffusion is hindered and simultaneous discharge of numerous cells might result in an accumulation of K^+ , or alternatively by disruption of gap junctions during dissociation [13]. Since ATP-sensitive K^+ (K_{ATP}) channels would shorten the APD, the longer APD suggests that the slices are not hypoxic.

Screening for drug-induced QT-interval prolongation is important for drug safety testing [14]. Long QT syndrome type 1, an autosomal disorder with syncope or sudden death due to ventricular tachyarrhythmias, is caused by mutations of the potassium channel gene KCNQ1 [15]. Linopirdine is a selective inhibitor of KCNQ channels [16] and KCNQ1 is expressed in the mouse heart during development [17]. We also demonstrate a prolongation of repolarization induced by linopirdine using embryonic slices. A significant additional advantage of tissue slicing is the possibility to study multi-cellular structures, which is demonstrated by the visualization of the spread of excitation wave using the MEA system [10].

Taken together, our results prove that viable tissue slices can be generated from embryonic hearts. The slices have morphologically conserved structures and stable electrophysiological characteristics. This makes it possible to conduct short and long-term experiments of cardiac function, and provides a powerful tool for drug safety studies. Our preparation will also facilitate studies of functional cross-talk between different cell types within the heart at a cellular and molecular level.

Acknowledgements

The work was supported by the Koeln Fortune Program / Faculty of Medicine, University of Cologne. We thank M. Breitbach, P. Sasse and Dr. B. Freedman for helpful comments on the manuscript.

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