Neonatal Murine Heart Slices. A Robust Model to Study Ventricular Isometric Contractions

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Key Words

Tissue slices • Murine myocardial contraction • Murine sarcoplasmic reticulum • Electrophysiology • Beta-adrenergic stimulation

Abstract

Background/Aims: Cardiac function is increasingly studied using murine models. However, current multicellular preparations to investigate contractile properties have substantial technical and biological limitations and are especially difficult to apply to the developing murine heart. **Methods:** Newborn murine hearts were cut with a vibratome into viable tissue slices. The structural and functional integrity of the tissue was shown by histology, ATP content and sharp electrode recordings. **Results:** Within the first 48 hours after slicing structure remained intact without induction of apoptosis. ATP concentrations and action potential parameters were comparable to those of physiological tissue. Isometric force measurements demonstrated a physiological force-frequency relationship with a 'primary-phase' negative force-frequency relationship up to 1-2 Hz and a 'secondary-phase' positive force-frequency relationship up to 8 Hz. (-)-Isoproterenol (10^{-6} mol/l) increased active force to $251 \pm 35\%$ ($n=15$) of baseline values and shortened relaxation times indicating a preserved beta-

adrenergic regulation of contraction. Changes of the force-frequency relationship after application of ryanodine and nifedipine indicated functionality of calcium release from the sarcoplasmic reticulum and of L-type calcium channels. **Conclusion:** Generation of viable, physiological intact ventricular slices from neonatal hearts is feasible and provides a robust model to study loaded contractions.

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Introduction

Murine models are increasingly used to study cardiac development, function and pathology. The ease to create genetic modifications has made this species popular [1]. Contractility is the major function of the heart. However, the small size of the murine heart poses methodological challenges, especially for the assessment of contractility during prenatal and early postnatal development. Frequently, optical methods are used that estimate contractility indirectly by measuring shortening of cell or sarcomere length of isolated unloaded cells [2]. However, despite a remarkable consistency in data from such unloaded cell-contraction experiments, externally loaded force development and shortening have turned out to be

related but not equivalent indices of contraction [2]. In addition, though many questions can be addressed on a molecular or cellular level, the importance of systems biology, i.e. holistic studies that take into consideration the complex interplay between different cell types is emerging. Therefore, not only for force measurements, multicellular preparations with maintained cell-cell interactions regain significance for cardiovascular research [3-7].

Despite this unquestionable importance for research, investigations on a tissue level pose several methodological challenges [8]. Optimally, the heart is supplied with oxygen and metabolites *via* the preexisting vascular system as for example in the Langendorff heart [8] or in arterially perfused wedges [9]. Since arterially perfused wedges have not yet been described for mice, the stability of the existing tissue preparations is largely dependent on diffusion [10]. Formerly, non-physiologically low temperatures and stimulation frequencies were used to reduce metabolic demands. Today, ultra thin right ventricular trabeculae are used to minimize diffusion length [11]. Since trabeculae are missing in the left ventricle, the clinical relevance of these studies is limited. In addition, since ultra thin trabeculae occur in a rather low frequency, measurements typically require on average two to three mice for a single successful experiment [4, 12]. Studies in embryonic or neonatal mice are even more challenging with only very few studies investigating newborn mice using muscle strips from right ventricular free walls [12].

Therefore, we studied whether reducing diffusion length by cutting myocardial tissue into thin slices would be an alternative and perhaps more convenient approach to generate multicellular preparations for force measurements. Slicing of cardiac tissue, has originally been described for toxicological studies [13] and has become an established procedure to assess cardiac metabolism [14]. Burnashev et al. [15] were the first to describe patch clamp recordings of myocardial slices in rats. However, patch-clamping turned out to be technically difficult in slices and some of these first preparations showed signs of tissue injury such as non-physiological resting membrane potentials. Recently, we could show that with an improved protocol viable tissue slices can be generated from late-stage murine embryonic hearts [16] and even adult hearts [17] allowing electrophysiological studies under physiological conditions. In this study, we demonstrate that neonatal preparations are morphologically and functionally intact and suitable as an alternative to current models to measure loaded contractions in mice.

Materials and Methods

Tissue harvesting and slicing

Experiments were carried out according to the Principles of Laboratory Animal care (NIH publication No. 86-23, revised 1985) as well as the current version of the German law on the Protection of Animals. Neonatal mice were sacrificed on postnatal day 3-4. Isolated hearts and slices were placed in cold (4°C) Ca²⁺-free Tyrode's solution (composition in mmol/l: NaCl 136, KCl 5.4, NaH₂PO₄*2H₂O 0.33, MgCl₂*6H₂O 1, glucose 10, HEPES 5, 2,3-butanedione monoxime 30; pH 7.4 adjusted with NaOH) that was continuously bubbled with pure oxygen; CaCl₂ was added as indicated. The effects of a brief exposition of 2,3-butanedione monoxime have been shown to be reversible [18]. All substances were purchased from Sigma Chemical Co. (Munich, Germany) unless otherwise stated. After removal of the atria with a scalpel, 4% low-melting agarose (Roth, Karlsruhe, Germany) dissolved at 70°C and maintained liquid at 34°C was poured over the hearts, chilled and allowed to solidify on ice. A cubiform block containing the heart was cut with a scalpel. These agarose-embedded hearts were fixed onto a steel specimen holder with cyanoacrylate glue (Pattex, Henkel, Germany) with the apex of the heart pointing upwards. Both ventricles were sectioned with steel blades (Campden Instruments, Leicester, England) orthogonally, i.e. along the short axis, into 300 µm thick slices with a vibratome (VT1000S, Leica Microsystems, Germany).

In contrast to preparations designed to study functional integration of transplanted cells (slice thickness: 150 µm) [17], the slice thickness of 300 µm turned out to be most useful for force measurements. After 30 minutes in cold Tyrode's solution (0.9 mmol/l Ca²⁺), slices were allowed to recover in Iscove's medium (1.5 mmol/l Ca²⁺) supplemented with 20% fetal calf serum (FCS) and kept in the incubator (21% O₂, 5% CO₂, 37°C) for up to twenty hours.

Histology

For histological and immunohistochemical examinations 7 µm thick cryosections were prepared. Immunohistochemistry was performed as described [16] using rabbit anti-active-caspase-3 antibody (1:500, Pharmingen, San Diego, Ca, USA) as the primary antibody in slices fixed with 4% paraformaldehyde for 120 minutes at room temperature. Slices were fixed for 30 minutes in 70% methanol at -20°C for immunostaining against titin (1:100, T11), a giant sarcomeric protein to identify cardiomyocytes, and vinculin (1:200, HVIN1), a focal adhesion protein with a major role in the transmission of contraction force.

ATP assay

ATP was quantified by a luciferase driven bioluminescence assay (ATP-Bioluminescence Assay Kit CLS II, Roche, Germany). Slices were put in sonification solution (ethanol 70% v/v, 2 mmol/l EDTA) immediately frozen in liquid nitrogen and stored at -80°C until further processing. Frozen slices were homogenized by sonification and mashed on ice before transfer into standard phosphate buffered saline to dilute ethanol and EDTA that might disturb the luciferase reaction.

Fig. 1. Mounting of tissue slices. (a) The tissue slice is spiked onto the glass pipette. (b) The glass pipette is broken. (c) The lumen of the capillary is imposed on the hooks enabling the slice to be pushed down.

Finally, luciferase reagent was added and luminescence was measured (Sirius Luminometer V2.2). Protein content was used as reference and was determined with a protein assay based on the method of Bradford (BioRad Protein Assay, BioRad Laboratories).

Action potential recordings

For conventional microelectrode recordings of transmembrane action potentials (APs) sharp glass electrodes filled with 3 mol/l KCl (tip resistance: 20–30 M Ω) were used. Signals were amplified by a SEC-10LX single electrode clamp amplifier (npi electronic, Tamm, Germany) and acquired with the PULSE software (HEKA, Lambrecht/Pfalz, Germany). AP parameters were analyzed offline with the Mini Analysis software (Synaptosoft, Fort Lee, NJ, USA). All recordings were performed at 37°C in Tyrode's solution (1.8 mmol/l Ca²⁺). A constant beating frequency of 2 Hz was assured by a stimulation system described elsewhere [19].

Force measurements

Mounting the tissue slices for force measurements was done with the help of glass tubes (Fig. 1). Thin wall borosilicate glass capillaries (outer diameter: 1 mm, inner diameter: 0.75 mm; World Precision Instruments, Berlin, Germany) were pulled manually in the flame of a Bunsen burner to create glass tubes with a peak and a thinned part (outer diameter: 0.4 mm, inner diameter: ca. 0.25 mm). The left ventricle of the tissue slice is spiked onto the glass pipette in a beaker filled with agarose covered by medium with the help of a stereo microscope and positioned at the thinned part by pushing the capillary into the agarose (Fig. 1a). The glass capillary is broken just below the tissue slice using a forceps (Fig. 1b). After the lumen of the broken capillary has been imposed on the hooks of the force transducer, the tissue slice is pushed gently onto the hooks using a custom-made fork-like slider (Fig. 1c). The hooks are made of steel needles (outer diameter 0.22 mm, Hwato, Suzhou

Medical Appliance Factory, China) bent to a J-shape connected to an isometric force transducer (Scientific Instruments, Heidelberg, Germany). Preparations were field stimulated by silver electrodes (0.5–10 Hz, 5–15 V, stimulus pulse duration: 5 ms) connected to a custom-made stimulator. The preparation was maintained at 37°C and immersed in a dish filled with Iscove's modified Dulbecco's medium without serum (1.5 mmol/l Ca²⁺). Length was increased stepwise to the length of maximal force development ($L_{\max}$) during continuous stimulation at 4 Hz.

Cross sectional area of cryoslices was determined by plane geometry from representative slices fixed while stretched to $L_{\max}$. In addition, cross sectional areas were determined by plane geometry of microphotographs of slices that were flattened down by a cover slide assuming a slice thickness of 300 μ m.

Electrical stimuli and analog signals from the force transducer (KG7A; range: 0–5 mN, resolution 0.2 μ N, resonance frequency 250–300 Hz) were amplified with a bridge amplifier (BAM7C; Scientific Instruments, Heidelberg, Germany), analog signals were transferred to an A/D board and recorded as well as analyzed using the software DasyLab version 7.0 (National Instruments, Munich, Germany).

Statistical analysis

All data are expressed as means $\pm$ SEM. One-way ANOVA was performed for each group using Bonferroni's post-hoc test in case of equal variance, posthoc Dunnett T3 test was used if variances were unequal that was determined by the Levene procedure. Paired t-tests were done to calculate the effects of (-)-isoproterenol on twitch parameters, unpaired t-tests were used to compare AP parameters of day 0 with day 1. Statistics were calculated using SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA). P-values <0.05 were regarded as statistically significant.

Fig. 2. Histological and biochemical studies. (a-c) Structural integrity and the typical divergent fiber orientation with longitudinally oriented fibers restricted to the midwall as shown by (a) hematoxylin & eosin staining, (b) methylene blue staining and (c) immunostaining against titin (green), vinculin (red) and nuclear counterstaining with Hoechst 33342 (blue) (c). (d-e) Staining against active-caspase 3, a marker for induction of apoptosis, is negative immediately after slicing (d) and on the following day (e) but prominent in a control slice subjected to 4.5 hours of oxygen and glucose deprivation (f). (g) ATP measurements showing preserved energy metabolism on postoperative days 0 and 1.

Results

Histology, immunohistology and biochemistry

Conventional histology (hematoxylin & eosin, methylene blue staining) demonstrated the structural integrity of the slices. Due to the well-known differences in fiber orientation [20] the fibers of the ventricular muscle of the endocardial and epicardial surfaces were cut predominantly orthogonal whereas only the midwall contained fibers with a circumferential direction that were cut longitudinally (Fig. 2a-c). Immunostaining for active-caspase-3 showed absence of induction of apoptosis in all slices 0-12 hours after slicing and in the majority of slices on the first postoperative day whereas slices subjected to oxygen and glucose deprivation that served as a positive control showed an induction of apoptosis (Fig. 2d-f).

In line with the morphological studies, biochemical measurements indicated that the content of the ATP was

stable during the first and second postoperative day but declined on postoperative day 3 (Fig. 2g). Absolute values are comparable to recent literature data from mice (37.5 ± 7.1 nmol/mg protein [21]).

AP recordings

To characterize basic electrophysiological properties of the neonatal heart slices, transmembrane APs of cardiomyocytes ($n=33$ cardiomyocytes) from different slices ($n=7$) were recorded *in situ*. A constant stimulation frequency of 2 Hz was applied to avoid frequency-dependent changes of AP parameters. Parameters of APs recorded at day 0 and day 1 after preparation are shown in Table 1. All APs had a stable resting membrane potential, a fast phase 0 upstroke and lacked a pronounced plateau phase. Resting membrane potential, time to maximal depolarization (AP_{RISE}), maximal upstroke velocity (V_{max}), action potential duration at 50% repolarization (APD_{50}) and AP amplitude displayed only

Table 1. Action potentials (APs). Parameters (mean $\pm$ SEM) of APs measured in murine neonatal heart slices on the day of preparation (day 0) and on the following day (day 1). APA: action potential amplitude; $V_{\max}$: maximal dV/dt of the upstroke; 10-90% Slope: dV/dt from 10-90% of the upstroke amplitude; 10-90% Rise: time from 10-90% of the upstroke amplitude; $APD_{50/90}$: time from action potential maximum to 50%/90% of repolarization; RMP: resting membrane potential, n.s.: not significant.
^a P-value for comparison of day 0 versus day 1.

Parameter	Day 0 (n=19)	Day 1 (n=14)	P-value ^a
APA	87.5 $\pm$ 2.5 mV	80.5 $\pm$ 1.1 mV	n.s.
$V_{\max}$	110.1 $\pm$ 10.0 V/s	101.4 $\pm$ 9.2 V/s	n.s.
10-90% Slope	71.7 $\pm$ 7.0 V/s	67.4 $\pm$ 6.8 V/s	n.s.
10-90% Rise	1.1 $\pm$ 0.1 ms	1.1 $\pm$ 0.1 ms	n.s.
APD_{50}	18.6 $\pm$ 1.1 ms	22.6 $\pm$ 1.5 ms	0.034
APD_{90}	47.9 $\pm$ 2.6 ms	98.8 $\pm$ 4.5 ms	<0.001
RMP	-72.0 $\pm$ 1.7 mV	-68.9 $\pm$ 1.7 mV	n.s.

Fig. 3. Action potential (AP) recordings. (a,b) Application of 25 mmol/l KCl. (a) The temporarily raised concentration of extracellular K^+ led to an instant depolarization of the resting membrane potential and a decline of action potential amplitude (0: 18 seconds before, 1: 1 second after KCl application) up to a complete stop of electrical activity (2: 6 seconds after KCl) that is washed out completely (3: 34 seconds, 4: 132 seconds after KCl). (b) Corresponding changes of resting membrane potential (filled circles) and action potential amplitude (open squares). (c,d) Variation of stimulation frequency. (c) APs recorded at 2 Hz (gray trace) and 7 Hz (black trace) and (d) frequency-dependent changes of repolarization in an example showing a prolongation of APD_{50} (filled circles) and APD_{90} (open circles) with increasing frequencies. In a total of 8 experiments, APD_{50} was prolonged in all cases, while APD_{90} was prolonged in 4/8 cases and remained constant in 4/8 cases.

minor alterations between day 0 and day 1 after preparation. APD_{90} changed significantly from 47.9 $\pm$ 2.6 ms (day 0; n=19) to 98.8 $\pm$ 4.5 ms (day 1; n=14). Addition of KCl 0.5 mol/l to the bath solution, resulting in a final bath concentration of approximately 25 mmol/l led to a depolarization of the resting membrane potential from -67.8 $\pm$ 1.6 mV to -20.3 $\pm$ 2.3 mV and a halt of electrical and contractile activity (n=9). The effect was reversible after reperfusion with Tyrode's solution (see Fig. 3a,b).

The effect of a variation of the stimulation frequency was analyzed in 8 experiments (example shown in Fig. 3c,d). APD_{50} increased in all cases, while APD_{90} increased in 4/8 and remained constant in 4/8 slices.

Isometric force measurements

In order to investigate the usefulness of this preparation to perform isometric force measurements, we analyzed basic properties of the ventricular rings with

Fig. 4. Relationship between stimulation rate and developed force. (a) Averaged force frequency relationship for preparations measured at postoperative day 0 or day 1 in murine ventricular tissue slices at 37°C (n as indicated) 90 s after frequency was changed. Data were compared individually with the force measured at 6 Hz; $[Ca^{2+}]_0 = 1.5$ mmol/l. Results are expressed as means $\pm$ SEM, asterisks indicate statistical significance ($p < 0.05$). (b-d) Active stress development in a typical slice during variation of stimulation rates. (e) Overlaid and normalized traces acquired at different stimulation rates show a faster relaxation at higher stimulation frequencies.

respect to cardiac muscle contraction: The average maximal inner diameter of the ventricular ring amounted to 2.2 ± 0.1 mm (range: 0.7-2.7 mm; $n = 16$) when the developed force peaked. In two stretched and subsequently cryosliced vibratome slices the estimated cross sectional area was 0.44 ± 0.1 mm². In typical slices that were not stretched the value calculated by plane geometry of microphotographs assuming a slice thickness of 300 μm was 0.64 ± 0.05 mm² ($n = 12$).

Fig. 5. Beta-adrenergic stimulation. (a) Effect of (-)-isoproterenol on the twitch amplitudes for preparations measured at postoperative day 0 ($n = 7$) or day 1 ($n = 8$) in murine ventricular tissue slices at 37°C, $[Ca^{2+}]_0 = 1.5$ mmol/l. Results are expressed as mean $\pm$ SEM, asterisks indicate concentrations with significant differences between day 0 and day 1. (b-d) Active stress development in a typical slice during variation of (-)-isoproterenol concentrations. (e) Overlaid and normalized traces acquired from a typical slice at different concentrations of (-)-isoproterenol show a faster relaxation at higher concentrations of (-)-isoproterenol.

We could show that when length was increased stepwise the stretch-induced increase of passive tension was accompanied by an increase of active tension until the length of maximal force was reached (positive length-tension relationship).

The average developed force at a stimulation rate of 6 Hz was 148 ± 17 μN ($n = 16$). The ratio of active to passive force was 0.59 ± 0.1 (range: 0.26-1.35; $n = 14$). A further increase of the length of the preparation beyond

Table 2. Beta-adrenergic stimulation. Twitch parameters of isometric contractions measured on postoperative day 0 or 1 before and with (-)-isoproterenol (10^{-6} mol/l) (n=15). Mean $\pm$ SEM. Paired t-test.

parameter	baseline	(-)-isoproterenol	p-value
developed force [μ N]	95.5 $\pm$ 18.0	250.8 $\pm$ 35.3	0.0001
time of maximum [ms]	66.8 $\pm$ 2.3	61.4 $\pm$ 1.4	0.0036
contraction velocity [mN/s]	3.1 $\pm$ 0.6	8.9 $\pm$ 1.2	0.0001
relative contraction velocity [%]	32.9 $\pm$ 0.7	35.5 $\pm$ 0.5	<0.0001
relaxation velocity [mN/s]	-1.8 $\pm$ 0.3	-7.1 $\pm$ 0.8	<0.0001
relative relaxation velocity [%]	-19.6 $\pm$ 0.8	-28.9 $\pm$ 1.1	<0.0001

Fig. 6. Effects of ryanodine and nifedipine on force-frequency relationship. (a,c,e) Nifedipine (0.1μ M) reduced amplitudes predominantly at high frequencies (b,d,f), ryanodine mainly at low frequencies. The high initial amplitudes observed after switching to a lower stimulation rate (c,d) are maintained after application of nifedipine (e) but abolished after ryanodine (f). Mean $\pm$ SEM, n=3 for nifedipine, n=5 (4 for 0.1 Hz) for ryanodine.

the length of maximal force resulted in a decrease of the developed force that was partially reversible.

While slices generated from late-stage embryonic hearts usually are spontaneously active and those prepared from adult murine hearts are not, only some of the neonatal slices exhibited spontaneous beating. Up to now, our data

on tissue viability argue against the assumption that spontaneous automaticity would be an indicator of tissue injury. Those with spontaneous contractions exhibited a beating frequency of 1.1 ± 0.1 Hz (range: 0.6 – 1.7 Hz). Testing of the force frequency relationship revealed that the developed force of the tissue slices was lowest with

a stimulation frequency of 1-2 Hz and continuously increased with an increase of the stimulation frequencies up to 8 Hz, the highest stimulation rate routinely used (Fig. 4a-d). The increase of the twitch amplitude with increasing beating rates was accompanied by a faster relaxation (Fig. 4e). In some experiments an alternans pattern occurred during stimulation with high frequencies (8-10 Hz). There was clear evidence for an increase of the force with lower frequencies, and after short periods of rest (<30 seconds) there was a pronounced increase of force of contraction of the first beat upon restimulation (post-rest potentiation).

Beta-adrenergic stimulation with (-)-isoproterenol during a stimulation with 4 Hz resulted in a dose-dependent increase of the developed force to $126 \pm 6\%$ at 10^{-8} mol/l, $287 \pm 37\%$ at 10^{-7} mol/l and $352 \pm 52\%$ at 10^{-6} mol/l ($n=15$) (Fig. 5a,b). Compared with results from postoperative day 0 ($n=7$), those of day 1 ($n=8$) showed a diminished increase due to (-)-isoproterenol at the later time point (Fig. 5a). With (-)-isoproterenol (10^{-6} mol/l) twitch parameters on both days showed a significantly faster contraction velocity and a faster relaxation, even when the results were normalized for the increase of amplitude induced by (-)-isoproterenol (Fig. 5c, Table 2).

Nifedipine ($0.1 \mu\text{mol/l}$) reduced twitch amplitudes primarily during stimulation at higher beating rates (Fig. 6a,c,e) while amplitudes increased during stimulation rates below 1 Hz. The latter might be due to a nifedipine-induced prolongation of the time until steady-state conditions were achieved.

In contrast, ryanodine ($0.1 \mu\text{mol/l}$) resulted in a reduction of the amplitude that was more pronounced at lower frequencies. In addition, ryanodine completely abolished the potentiation of the first beats after switching from higher to lower stimulation rates (Fig. 6b,d,f).

Discussion

Multicellular preparations are an indispensable tool for biomedical research and the increasing use of mice as experimental models in biomedical research [1] necessitates to adopt investigative techniques to this species. However, isometric force measurements in mice using thin trabeculae or papillary muscles remains difficult and is limited to adult animals [4].

In addition, though the study of the adult murine heart already yielded relevant information, differences between the adult murine and human myocardium are clearly visible when comparing the duration of the APs. Due to

substantial differences with respect to the expression and functional roles of potassium and other ion channels the action potential duration (APD) of the human ventricular myocardium (APD at 50% repolarization (APD_{50}): 190 ms [22]) is about forty times longer than that reported for the adult murine heart (APD_{50} : 3-10 ms [23, 24]). In contrast, APD of neonatal (APD_{50} : 18 ms [23], 20 ms, this study) or late-stage embryonic hearts (APD_{50} : 116 ms [16]) and their ion channel composition [24, 25], are far closer to those of the human heart. Therefore, a model that would ease the generation of preparations and could even extend the investigations to the neonatal and late-stage embryonic age would have a significant impact on experimental work. We aimed to analyze the viability of neonatal murine ventricular tissue slices and to test whether these would be suited for the investigation of contractility as well.

Conventional histology demonstrated the structural integrity of the slices. Missing immunoreactivity with active-caspase-3 on postoperative days 0 and 1 indicated that apoptosis is not yet induced during the first 48 hours after slicing. ATP levels that were within the physiological range reported previously [21] confirmed a preserved energy metabolism.

Recordings of transmembrane APs were performed to judge functional integrity electrophysiologically. The measured resting membrane potential is similar to that of isolated neonatal cardiomyocytes [26]. This indicates the maintenance of the ion gradient as an indirect indicator of ATP hydrolysis since it is maintained primarily by the activity of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ [27]. Even if the amplitude of the action potential ($84.5 \pm 1.6\text{mV}$ vs. 95mV [26]) as well as the maximal upstroke velocity ($106 \pm 6.9 \text{ V/s}$ versus $182 \pm 49 \text{ V/s}$ [23]) were slower than those of isolated neonatal cardiomyocytes, the upstroke of the action potential was steep indicating that it is largely dependent on voltage-dependent sodium currents which is in line with our previous observation of a physiological conduction velocity and spread of excitation within the sliced tissue [28]. Further electrophysiological evidence for a robust viable state was provided by demonstrating the recovery from the depolarization induced by a high extracellular potassium concentration.

Characteristic for neonatal ventricular cardiomyocytes repolarization was faster than in embryonic cells but slower than in adult cardiomyocytes indicating developmental changes of potassium channel expression [23]. Absolute values for the APD_{50} matched previous reports [23, 29], whereas especially on day 1 APD_{90} was longer than reported in other studies [23, 29].

The observed prolongation of the repolarization phase with increasing frequency again is in line with similar observations in adult mouse hearts mounted on a Langendorff apparatus [30].

Since histological, biochemical and electrophysiological data demonstrated the physiological condition of the slices we performed isometric force measurements to characterize the contractile properties of this preparation. The slices turned out to be a useful and robust preparation since it allowed reproducibly the generation of numerous preparations from a single heart and even relevant measurements one day after slicing. Though absolute values for the developed force and the ratio of active to passive force were lower than known from trabeculae or papillary muscles [5], qualitative data strongly supported the view that the slices were in a viable, physiological state. In contrast to many studies which used the reduced metabolic need at reduced temperatures, we measured the force-frequency relationship at 37°C and still showed a primary negative phase below and a secondary positive phase above 1-2 Hz for frequencies of at least up to 8 Hz, the highest frequency that was tested routinely. A negative force-frequency relationship is frequently discussed to indicate the quality of the preparation and the sufficiency of nutrient supply [11]. Our data are in line with previously reported carefully designed studies of trabeculae or papillary muscles [3, 5] and in clear contrast to observations from tissue during heart failure [31]. In line with previous studies relaxation times decreased with increasing stimulation rate [3, 5].

We found evidence of a physiological response to β -adrenergic stimulation, i.e. an increase of the developed force. Down-regulation of the effects of isoproterenol was observed within the first 24 hours after the slicing procedure despite a preserved basal contractility. This catecholamine desensitization might be caused by the depletion of noradrenaline stores that is known to occur after isolation procedures [32]. The faster relaxation induced by isoproterenol has been shown to be related to the function of the sarcoplasmic reticulum since it can be blocked by ryanodine [33]. In addition, the reduction of the amplitude induced by nifedipine especially at high frequencies that can be explained by the use-dependency of the L-type calcium channels [34] and stresses the previously reported importance of this ion channel in murine myocardium [6]. The force reduction by ryanodine at low stimulation rates were identical to the observations made by Stuyvers et al. [6] and indicates that, in addition to frequency-dependent Ca^{2+} sensitization of the myofilaments [11], the relationship between force

and frequency at low frequencies is largely dependent on calcium sequestration by the sarcoplasmic reticulum [6]. These results indicate an important role for L-type calcium channels and sarcoplasmic reticulum and provide further evidence for the well preserved cardiac functionality after slicing [35].

It is known that cardiac fiber arrangement as counter-wound helices encircling the ventricles is crucial for achieving sufficient ejection fractions [36]. Fiber direction is oriented predominantly in the base-apex direction on the epicardial and endocardial surfaces and is rotated to a circumferential direction in the midwall only [20]. This fiber architecture will result in a special twisting motion during systole in the whole heart. In contrast, only a limited number of fibers of the tissue slice actually develop force in the direction recorded by the force transducer and will lead to a rather low number of myofibrils that are stretched to the optimal sarcomere length of $\sim 2.2 \mu\text{m}$ [4]. Nevertheless, this should not limit the usefulness of the preparation *per se* as long as no comparison with data obtained with other techniques is required.

In conclusion, we consider the slicing technique to be a valuable and robust tool to create viable tissue slices that are well suited to perform isometric force measurements of neonatal ventricular myocardium. Future applications can be expected to be studies on contractile properties of genetically modified mice, especially if pre- or perinatally lethal.

Abbreviations

AP (action potential); APD_{50} (time from action potential maximum to 50% of repolarization); APD_{90} (time from action potential maximum to 90% of repolarization); AP_{RISE} (time to action potential maximum); V_{max} (maximal upstroke velocity); APA (action potential amplitude).

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