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Original article

To establish a pharmacological experimental platform for the study of cardiac hypoxia using the microelectrode array

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ABSTRACT

Introduction: Simultaneous recording of electrical potentials from multiple cells may be useful for physiological and pharmacological research. The present study aimed to establish an *in vitro* cardiac hypoxia experimental platform on the microelectrode array (MEA). **Methods:** Embryonic rat cardiac myocytes were cultured on the MEAs. Following ≥90 min of hypoxia, changes in lactate production (mM), pH, beat frequency (beats per min, bpm), extracellular action potential (exAP) amplitude, and propagation velocity between the normoxic and hypoxic cells were compared. **Results:** Under hypoxia, the beat frequency of cells increased and peaked at around 42.5 min (08.1 ± 3.2 bpm). The exAP amplitude reduced as soon as the cells were exposed to the hypoxic medium, and this reduction increased significantly after approximately 20 min of hypoxia. The propagation velocity of the hypoxic cells was significantly lower than that of the control throughout the entire 90+ min of hypoxia. The rate of depolarisation and Na^+ signal gradually reduced over time, and these changes had a direct effect on the exAP duration. **Discussion:** The extracellular electrophysiological measurements allow a partial reconstruction of the signal shape and time course of the underlying hypoxia-associated physiological changes. The present study showed that the cardiac myocyte-integrated MEA may be used as an experimental platform for the pharmacological studies of cardiovascular diseases in the future.

1. Introduction

Cultured heart cells are useful for the study of cardiac pathology. Indeed, isolated rat cardiac myocytes have been used as an experimental model in the study of anoxic cell injury since the early 1980s (Rajs & Harm, 1980). One major advantage is that these cells can be easily obtained from embryonic or neonatal animals, and they provide the means of studying cellular morphology, biochemistry, and electrophysiological characteristics of the mammalian heart (Chlopcikova, Psotova, & Miketova, 2001). Thus far, cultured cardiac myocyte models have proven to be very useful for the study of hypoxic injury (Orita et al., 1995; Liu, Chen, Yang, Cheng, 2001; Bollensdorff, Knopp, Biskup, Zimmer, & Benndorf, 2004; Eigel, Gursahani, & Hadley, 2004) and hypoxic preconditioning (Webster, Discher, & Bishopric, 1995; Hausenloy, Yellon, Mani-Babu, & Duchen, 2004). The use of isolated cardiac myocyte models to study cardiac functions has been reviewed (Diaz & Wilson, 2006). The effects of cardiac hypoxia are often correlated with the functionality of the cells before and after hypoxic episodes using a patch-clamp approach (Liu et al., 2001; Bollensdorff et al., 2004). There is no doubt that the patch-clamp technique can yield important information on the cellular electrophysiology of a few cells. This approach, however, cannot provide a comprehensive picture of cell-to-cell signal propagation characteristics, and continuous long-term recording is not practical — the microelectrode arrays (MEAs) may provide the answers to these problems. The rationale behind the use of MEAs is based on the integration of multiple cells on microchips in order to detect changes of extracellular electrophysiological signals. This system enables the recording of many cells simultaneously, which is useful when a global view of a population of cells is desired as in the case of cardiac hypoxia.

Our previous studies have demonstrated the potential application of cultured embryonic cardiac myocyte-integrated field effect transistor arrays in pharmacological bioassay (Ingebrandt, Yeung, Krause, & Offenhausser, 2001; Yeung, Ingebrandt, Krause, Offenhausser, & Knoll, 2001; Ingebrandt, Yeung, Staab, Zetterer, & Offenhausser,

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2003). Parallel to this project, an MEA system that uses 64 planar gold microelectrodes for signal recording has been developed (Krause, 2000; Yeung et al., 2001; Ecken et al., 2003; Zhang et al., 2005). The characterisation of electrophysiological recordings of embryonic heart activity using the MEA has been described (Reppel et al., 2004).

Through significant improvement over the past few years, the present system has much higher signal-to-noise ratio and is much more reliable than our previous system (Wrobel et al., 2007). It is thus ideal for observing the electric activity of cells over a relatively long period of time. Furthermore, despite only extracellular signals are being recorded, this system is so sensitive that even changes in extracellular action potential signal shapes can be detected (Ingebrandt et al., 2001; Yeung et al., 2001; Yeung et al., 2007).

The purpose of this study was to establish an *in vitro* experimental platform of cultured cardiac myocytes on the MEA and to use this system to monitor the electrophysiological changes of the entire syncytium due to acute hypoxia. The obtained electrophysiological observations were compared with the presently known physiological changes, such as lactate concentration, pH, and osmolarity, of the heart under hypoxia. This study aimed to show that such a cell-integrated electronic system may be useful for a variety of pharmacological studies of heart.

2. Methods

2.1. Cell culture solutions and reagents

The standard culture medium was Ham's F10 medium, containing 10% (v/v) foetal bovine serum (FBS), 0.5% (v/v) insulin, transferrin, selenite (ITS) solution, 6 mM L-glutamine, and 2% (v/v) mixture of penicillin/streptomycin (5000 U/ml penicillin and 5 mg/ml streptomycin) adjusted to pH 7.4.

Cell culture reagents were obtained from Sigma: F10 Ham's (N6635), Hanks Balanced Salt Solution (HBSS, H6648), FBS (F7524), pen-strep mixture (P9096), L-glutamine (G7513), trypsin-EDTA (T4299), DNase II (D8764), ITS (I1884), fibronectin (F0635).

2.2. Cell preparation

Hearts of embryonic days 16–19 Sprague–Dawley rats were removed, minced, and placed into cold Ca^{2+}/Mg^{2+}-free HBSS. The chopped hearts were then pooled and washed several times to remove blood and other tissue fragments. After washing, the HBSS was replaced with 8 ml of 0.05% crude trypsin-EDTA. After 8 min incubation at 37 °C, the supernatant was discarded. The dissociation cycle then began with another 2 ml of trypsin for 8 min preceded by the addition of 100 µl DNase II solution (10,000 U/ml) for 1–2 min. The resulting supernatant was collected and added into the culture medium to stop trypsin digestion. This cell suspension mixture was centrifuged at 2000 rpm for 5 min. The pellet was resuspended using the standard culture medium. The above dissociation cycle was repeated four to five times. All procedures were carried out in sterile conditions.

The resultant cell suspensions were pooled and incubated for 2 h at 37 °C for the purpose of differential adhesion. This procedure allows fibroblasts to adhere to the culture dish preferentially over cardiac myocytes, thus increasing the myocyte-to-fibroblast ratio of the cell suspension. Approximately 32,000 to 48,000 cells were seeded onto each fibronectin-primed MEA surface.

2.3. Experimental setup

2.3.1. Microelectrode arrays

The MEA chips used for the extracellular recordings were first described by Krause (2000). The chips were manufactured on glass wafers (Borofloat 33, SCHOTT GLAS, Mainz, Germany) using standard optical lithography. The planar 64-channel gold MEAs (8×8) were designed with diameters of either 10 or 20 µm and a pitch of either 100 or 200 µm. In order to use the MEA several times, the chip surface was passivated by an oxide–nitride–oxide (ONO) layer deposited by plasma enhanced chemical vapour deposition (PECVD) consisting of 500 nm SiO_2, 500 nm Si_3N_4, and 100 nm SiO_2. Details of the fabrication and the flip-chip encapsulation processes have been previously described (Krause, 2000; Krause et al., 2000; Ecken et al., 2003).

2.3.2. Amplifier setup

The measurement setup for signal recordings with planar MEAs consists of a preamplifier and a main amplifier. In recent years, the pre-amplifier headstage of the system has been greatly improved, and it now offers high sensitivity with a large bandwidth (Zhang et al., 2005; Wrobel et al., 2007). This enables the recording of undistorted extracellular action potential signal shapes from cardiac myocyte cultures. The microelectrodes of the present setup are directly connected to a high impedance operational amplifier (Zhang et al., 2005; Wrobel et al., 2007). This configuration enables recordings with planar gold microelectrodes as small as 10 µm in diameter; therefore, signals from individual cells can be recorded. These signals are termed extracellular action potentials (exAPs) as opposed to the usually recorded field potentials from large, low-impedance microelectrodes using the commercially available MEA systems (www.multichannelsystems.com; www.plexoninc.com; www.med64.com). A detailed description of the pre-amplifier and its frequency characteristics can be found elsewhere (Wrobel et al., 2007). Data acquisition was carried out with our standard 64-channel amplifier in combination with an A/D card (PCI 6071E, National Instruments, Hong Kong) operated using the MED64 conductor 3.1 software (Alpha MED Sciences Co. Ltd., Japan). This main amplifier and software are similar to the setup used in the study by Ecken et al. (2003).

2.3.3. Hypoxia recording chamber setup

The recording setup consisted of a headstage amplifier with a Perspex incubation chamber placed on top of it to reduce air movement and excessive evaporation during experimentation (Fig. 1a). An outer Perspex casing and a Faraday's cage were used to encompass the entire headstage amplifier to further eliminate ambient air current and external electromagnetic noise, respectively. A vibration-free unit, which is made up of alternate layers of solid steel and thick rubber, was placed under the headstage amplifier to prevent vibrations coming from the lab bench (Fig. 1b). The temperature was maintained at 37 °C throughout.

2.4. Induction of hypoxia

The hypoxic medium was prepared by gassing the normal medium with a mixture of 95% N_2 and 5% CO_2 for a minimum of 5 min, and the concentration of dissolved O_2 after gassing (0 to 0.5%) was verified using a waterproof hand-held dissolved O_2 meter (Eutech Instruments, Singapore). After taking a control recording of 60 s, the normal medium was replaced with the pre-warmed hypoxic medium. Because of a 5-min delay between changing the medium and returning the MEA chip back onto the headstage amplifier, the moment when the hypoxic medium was added to the cell-containing MEA was considered as 0 min. After this 5-min delay, the recording was resumed while exposing the entire system to the same pre-warmed and humidified hypoxic gas mixture throughout the recording period. A recording of 30 s was taken every 2 min for a minimum of 90 min of hypoxia. The results were compared with those obtained in normoxic condition (95% air and 5% CO_2).

2.5. Lactate release, pH and osmolarity measurements

Lactate is a by-product of carbohydrate metabolism. The amount of lactate production, which affects the physiological state of the cells in normoxic or hypoxic conditions, was evaluated colorimetrically. The lactate reagent and lactate standards (20, 40, 80, and 120 mg/L), both