Journal of Pharmacological and Toxicological Methods 59 (2009) 146–152

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

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

Chi-Kong Yeung [a,*], Frank Sommerhage [b], Günter Wrobel [b], Jessica Ka-Yan Law [c], Andreas Offenhäusser [b], John Anthony Rudd [a], Sven Ingebrandt [b,1], Mansun Chan [c]

[a] Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong (CUHK), Shatin, Hong Kong
[b] Institute of Bio- and Nanosystems — Bioelectronics (IBN-2) and Centre of Nanoelectronic Systems for Information Technology (CNI), Leo-Brandt-Str., Research Centre Jülich, D-52425 Jülich, Germany
[c] Department of Electronic and Computer Engineering, The Hong Kong University of Science and Technology (HKUST), Hong Kong

ARTICLE INFO

Article history:
Received 9 January 2009
Accepted 17 February 2009

Keywords:
Cardiac myocytes
Disease model
Extracellular action potential
Functional studies
Hypoxia
Microelectrode array

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,

* Corresponding author. Room 405R, Basic Medical Sciences Building, Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong. Tel.: +852 9533 2699; fax: +852 2603 5139.
E-mail address: chi-kong_yeung@lycos.com (C.-K. Yeung).
[1] Present address: Department of Informatics and Microsystem Technology, University of Applied Sciences Kaiserslautern, D-66482 Zweibrücken, Germany.

1056-8719/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.vascn.2009.02.005

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

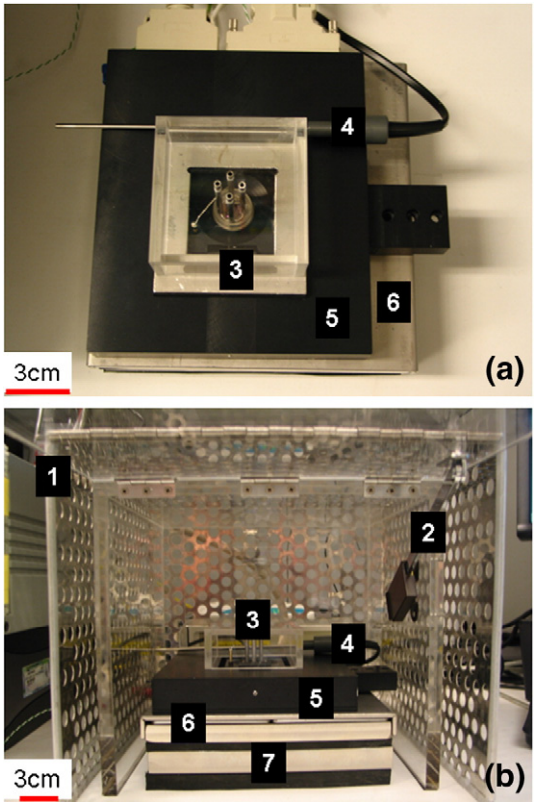

Fig. 1. The MEA recording setup. Top view of the (a) headstage amplifier and (b) the complete recording setup. The entire recording setup consists of a Faraday's cage (1), an outer protective Perspex casing (2), an inner Perspex incubation chamber (3), a thermocouple that measures the headstage temperature (4), the headstage amplifier (5), a thermostatically-controlled hot plate (6), and a vibration-free bench which is made up of alternate layers of solid steel and thick rubber (7).

purchased from SIGMA, were used to verify the accuracy of the readings. The enzymatic determination of lactate in the culture medium was read using multiplate reader (Dynex Opsys MR) at $\lambda = 550$ nm. The calculation of the final lactate concentration is given by: *Absorbance of TestSamples ÷ Absorbance of Standard × Concentra- (mM). The cells samples were analysed after 5, 10, 20, 40, 80, or 160 min of hypoxia, and the results were compared with those obtained from the normoxic control. pH and osmolarity measurements pre- and post-hypoxic insult were determined using a standard pH meter (Type 911 pH, Knick Portamess, Germany) and an osmometer (Osmomat 030, Gonotec, Germany), respectively.*

2.6. Measurements and analysis of results

The extracellular signals were analysed using a custom-designed software implemented in MATLAB® (Version 7.0, The MathWorks, USA). The results are expressed in terms of (i) beat frequency (beats per minute, bpm), which is a measure of functionality of the cultured cardiac cells; (ii) exAP amplitude (mV), which is the combined measurements of the positive peak of depolarisation (i.e. a measure of the rate of depolarisation) and the negative peak of the sodium (Na^+) signal (i.e. a measure of the extent of Na^+ entry); and (iii) propagation velocity (cm/s), which is a measure of conductivity among the cardiac cells. The means of these three parameters from all available channels of each MEA chip were calculated (i.e. $n = 1$) before obtaining the final means of all the chips from the same treatment group. All electrophysiological data are shown as mean ± S.E.M., and differences between treatment groups were compared using Student's unpaired *t*-test. The amounts of lactate present in the medium at different durations of hypoxia versus control were compared using Student's unpaired *t*-test; and the osmolarity and pH measurements, ± S.E.M., before and after hypoxic insult were compared using Student's paired *t*-test. In all cases, $P<0.05$ indicates a significant difference between values.

3. Results

The present study measured some well-known physiological changes that are associated with hypoxic insult in an attempt to correlate these changes with electrophysiological measurements obtained using the MEA.

3.1. The effect of continuous gassing

The normoxic culture medium usually contained 92.5% to 95.2% dissolved O_2 (8.30–8.47 mg/l). The concentration of dissolved O_2 was brought down to 0 to 0.5% following gassing of the culture medium with 95% N_2/5% CO_2 gas mixture for at least 5 min. If this anoxic solution was continuously gassed with this gas mixture, the amount of dissolved O_2 would remain below 1%. This was important as it appeared that atmospheric O_2 could diffuse back into this anoxic culture medium without continuous gassing, causing a steady rise in the amount of dissolved O_2 to approximately 5.0%, 11.6%, and 25.2% after 30 min, 60 min, and 90 min, respectively. While continuous gassing was carried out throughout the recording period and that the flow rate was kept very low, it could not be done directly over the medium so as to avoid excessive evaporation. As there was no guarantee that the medium would be completely free of O_2, it would be more appropriate to designate this medium and the condition of this study as 'hypoxic' rather than 'anoxic'.

3.2. Lactate concentration and pH

The progressive increase in lactate concentrations over 160 min of hypoxia (Fig. 2) was well correlated with a drop in pH. The pH of the medium remained stable when the cells were exposed to the normoxic gas mixture (7.47 ± 0.08 at 0 min versus 7.50 ± 0.04 at 90 min, $n = 5$), but it dropped to 7.26 ± 0.01 following 90 min of hypoxia ($n = 5$, $P<0.05$).

3.3. The effect of hypoxia on osmolarity

While normoxic (95% air/5% CO_2) or hypoxic (95% N_2/5% CO_2) gassing was not carried out directly on top of the cell-containing MEA chip during recording, gassing its adjacent area might still increase the rate of evaporation due to convection inside the incubation chamber. Elevated evaporation could lead to dehydration, and this would ultimately give rise to a hyperosmotic culture medium. As such,

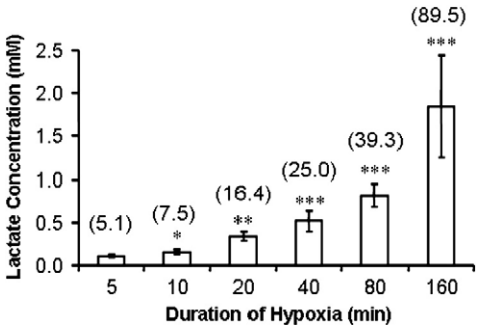

Fig. 2. The amount of lactate released from cells into the medium increased significantly after 10 min of hypoxia treatment (mM ± S.E.M.). Numbers in parentheses indicate the percentage increase of lactate at the corresponding time points. The results were analysed using Student's unpaired *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

osmotic pressure changes before and after recordings were recorded. Continuous (90 min) gassing increased the osmotic pressure of the medium from 290.4 ± 2.2 mosm (before any gassing, $n = 5$) to 302.2 ± 7.6 mosm (or 4.1%, $n = 5$) and 314.5 ± 18.0 mosm (or 8.3%, $n = 4$) under normoxic and hypoxic gassing, respectively. These results showed that while there were differences in osmolarity after gassing as well as between different gassing mixtures, but they were insignificant and that hypoxic gassing increased the culture medium osmolarity only marginally more than normoxic gassing did.

3.4. Electrophysiology of cardiac myocytes

Under normoxic conditions, the cultured cardiac myocytes had a basal beat frequency of 55.8 ± 9.1 bpm, an exAP amplitude of 0.4 ± 0.1 mV, and a propagation velocity of 7.1 ± 1.0 cm/s ($n = 10$). These measurements remained stable over 90+ min of normoxic recording (Fig. 3). These parameters displayed a particular pattern of change as the duration of hypoxia increased. The beat frequency of cells increased almost as soon as hypoxia had begun (Fig. 3a). The maximum beat frequency was observed at approximately 42.5 min (88.1 ± 3.2 bpm, $n = 10$), it then began to reduce gradually until it dropped below the basal level (42.0 ± 6.9 bpm at 100+ min, $n = 3$). The exAP amplitude of the cells reduced, albeit insignificant due to large standard errors, upon exposure to the hypoxic medium, and this drop increased after about 20 min of hypoxia. The propagation velocity of the hypoxic cells was significantly lower than that of the control throughout the hypoxic treatment, and this drop accelerated at just below 30 min. Both exAP and propagation velocity reached their respective lows at about 75 to 80 min (Fig. 3b and c, respectively).

The extracellular signal shapes of cardiac myocytes are composed of several signal components, and the interpretations of signal shapes have been described (Yeung et al., 2007). These signal shape components are outlined as follows:

1. The fast up-spike is related to the depolarisation of the cell membrane. The amplitude of this peak is proportional to the first derivative of the time-dependent membrane voltage $V_M(t)$.
2. The fast down-spike is related to the Na^+ currents through the small cleft between the membrane and the sensor surface.
3. The slow negative signal component is mainly the result of calcium (Ca^{2+}) influx.
4. The slow positive signal is the result of the repolarising potassium (K^+) efflux.

The changes in the fast up-spike and fast down-spike were progressively attenuated, indicating the rate of depolarisation and the extent of Na^+ influx signal were reduced as a direct result of hypoxic insult (Fig. 4a). These changes also affected the exAP durations, with overall reductions between 6 and 22% over 60 min of hypoxia. The magnitudes of both Ca^{2+} influx and K^+ efflux signals also reduced but only very slightly.

The examination of the actual exAP signal shapes provided an even better visualisation of the physiological changes. Individual signal shapes showed that the overall rate of depolarisation of the cells was severely diminished, the extent of the Na^+ influx signal was gradually reduced, and the duration was progressively lengthened from 5 to 50 min of hypoxia. These changes indicated that the membrane potential was gradually becoming hyperpolarised over the course of hypoxic insult (Fig. 4b).

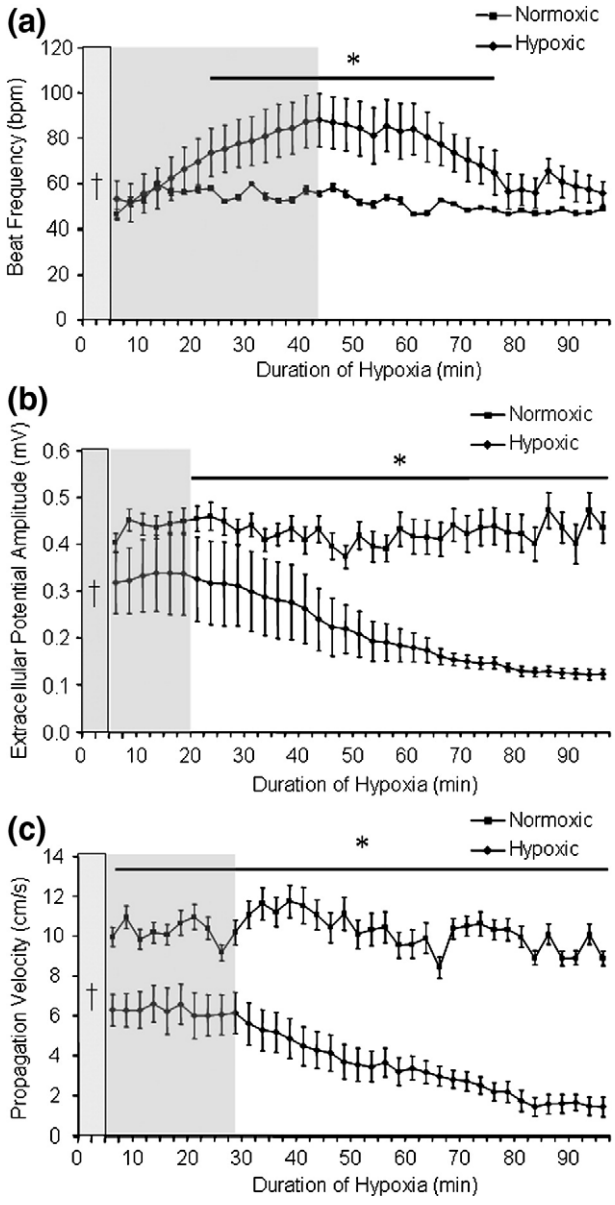

Fig. 3. Changes in beat frequency (bpm, a), exAP amplitude (mV, b), and velocity (cm/s, c), ± S.E.M., of cultured cardiac myocytes at different time points (0 to 90+ min) are shown. As it took time to change medium and to return the MEA chip back onto the headstage amplifier, there would be a 5-min delay prior to the actual start of recording (†denotes this 5-min delay between the change of hypoxic medium and the actual resumption of recording). The beat frequency was elevated up to 42.5 min of hypoxia; thereafter, it began to reduce and dropped below the normoxic value after 100+ min of hypoxia (not included in figure). The exAP amplitude reduced slightly as soon as the cells were exposed to the hypoxic medium, and this drop increased after about 20 min of hypoxia. The propagation velocity reduced significantly throughout the entire 90+ min of hypoxia; however, this reduction accelerated at just under 30 min. These time-dependent observations over the course of hypoxic insult are indicated by the shaded boxes. Both exAP and propagation velocity reached their respective lows at about 75 to 80 min. The results were analysed using Student's unpaired *t*-test (*$P < 0.05$).

4. Discussion

This study provides an extracellular electrophysiological profile of cultured rat cardiac myocytes under hypoxic insult over time with the aim of demonstrating the MEA can be used as an experimental platform for functional studies. Cultured cells are useful for biomedical research, and the use of primary neonatal rat cardiac myocytes as a functional model for physiological research has been reviewed (Chlopcikova et al., 2001). The combination of this physiological model with the present microelectrode system has provided a stable platform for the study of cardiac hypoxia, and this may be useful for pharmacological studies of the heart.

The heart is an energy demanding organ that can be easily damaged when oxidative phosphorylation is inhibited either due to depletion of adenosine triphosphate (ATP) or deprivation of oxygen. When either of these happens, ATP and creatine phosphate concentrations would

decrease; while the concentrations of adenosine diphosphate (ADP), adenosine monophosphate (AMP), and phosphoinositide (Pi) would increase (Halestrap, Clarke, & Khaliulin, 2007). During hypoxic or ischaemic insult, the heart ceases to beat because the contractile machinery is inhibited by the elevation of [ADP] and [Pi] as well as a reduction of pH as a direct result of an accumulation of lactate (Halestrap, Kerr, Javadov, & Woodfield, 1998; Suleiman, Halestrap, & Griffiths, 2001; Halestrap, Clarke, & Javadov, 2004). The elevation of lactate concentration and its associated reduction in pH over the course of hypoxia were verified in this study.

Continuous measurements show that atmospheric O_2 could diffuse back into the original 'anoxic' medium; therefore, continuous hypoxic gassing was necessary in order to maintain a low O_2 environment inside the inner Perspex incubation chamber. Although this procedure still elevated the osmolarity of the bathing medium in either normoxic or hypoxic conditions over time, it did not mount to a significant difference from the control (i.e. before any form of gassing). Furthermore, 90 min of normoxic recordings of all three physiological parameters (i.e. beat frequency, exAP amplitude, and propagation velocity) were relatively stable, indicating that this marginal change in osmolarity did not have any significant impact on the electrophysiological responses.

The present electrophysiological measurements have demonstrated some characteristic cellular phenomena as a result of hypoxia:

(1) The beat frequency of the cells was initially elevated (up to 42.5 min) followed by a gradual reduction (Fig. 3a). This seeming anoxia-provoked tachycardia has also been found in isolated spontaneously beating embryonic chick hearts (Sedmera, Kucera, & Raddatz, 2002). While the beat frequency was lower than the basal level at the end of the 90+ min of hypoxia and that the amplitude and conduction velocity were both reduced to very low levels, a complete cessation of beat did not occur despite prolonged hypoxic insult. The Na^+ current in the heart consists of two components: a very slowly inactivating persistent Na^+ (I_{Na} P) and a transient Na^+ (I_{Na} T) (Saint, 2008). It has been demonstrated that the I_{Na} P current is enhanced during hypoxia (Ju, Saint, & Gage, 1996; Barry, 2006; Saint, 2006; Wang, Ma, Zhang, Luo, 2007; Saint, 2008), while the I_{Na} T current is reduced (Wang et al., 2007). It is this I_{Na} P that contributes to Na^+ loading and the resultant Ca^{2+} overload and arrhythmias (Belardinelli, Shryock, Fraser, 2006; Saint, 2008). These changes in I_{Na} P and Ca^{2+} overload could have accounted for the initial increase in beat frequency observed. Furthermore, Ca^{2+} overload can contribute to ATP depletion via the activation of Ca^{2+}-dependent ATPases (Barry, 2006). This reduction in ATP formation would subsequently give rise to the opening of ATP-dependent potassium (K_{ATP}) channels and thus a reduction in mechanical activity of the myocytes. These physiological changes would not only have accounted for the eventual reduction in beat frequency, but also

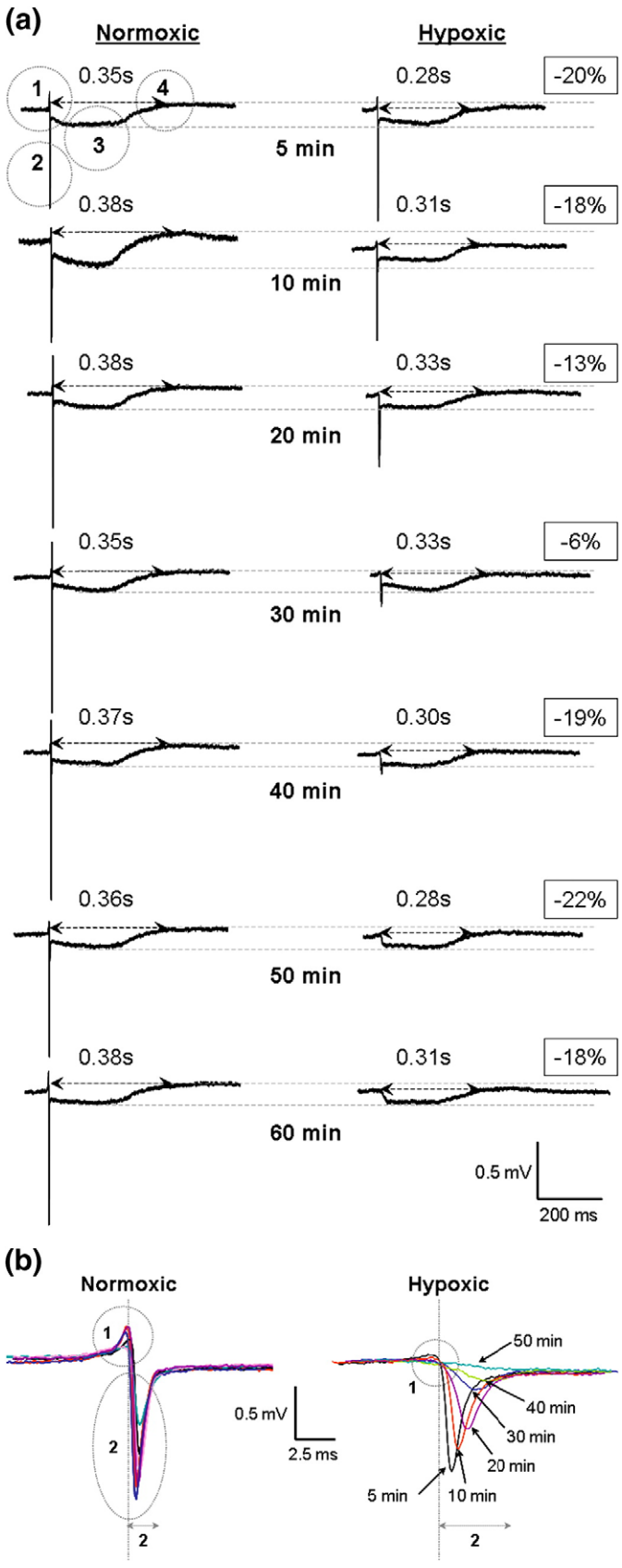

Fig. 4. (a) The figure shows the characteristic signal shape components (1 to 4 indicated at 5 min normoxia) and the changes in these shapes under normoxia or hypoxia from 5 to 60 min: (1) the fast up-spike is indicative of the rate of membrane depolarisation; (2) the fast down-spike is related to the sodium ion current; (3) the slow negative signal is mainly due to the transient calcium influx signal; and (4) the slow positive signal is due to the repolarising potassium efflux signal. The overall signal shape reductions were correlated with the duration of hypoxia, with progressive reductions in both signal components (1) and (2). The magnitudes of the combined signal components (3) and (4) under hypoxic condition, albeit not substantial, were also smaller than those obtained from the normal cells at corresponding time points (horizontal parallel dotted lines). The overall exAP duration (in second) at a particular point of hypoxia is indicated above each trace (actual durations are marked using lines with arrows at both ends), and the net differences between normoxic and hypoxic cells at corresponding time points are also shown as percentages (boxes). (b) The figure shows that the rates of depolarisation (component 1) from 5 to 50 min were severely diminished even after just 5 min of hypoxia. The sodium signal magnitudes reduced and their durations lengthened progressively from 5 to 50 min of hypoxia (component 2), indicating the electrochemical gradient of sodium during these periods was gradually being reduced due to hypoxic insult. Normoxic traces across the same time points are shown as comparison. Signal components (3) and (4) cannot be seen at this time scale.

the reductions in exAP amplitude, exAP duration, and conduction velocity observed in the present study (see below).

(2) The exAP amplitude, which is the combined measurements of the rate of depolarisation and the extent of Na^+ entry, reduced after approximately 20 min of hypoxia (Fig. 3b); and this reduction was associated with a shortening of exAP duration (Fig. 4a), which reflects the status of ionic fluxes. The reduction in exAP amplitude and the accompanied shortening of exAP duration have also been demonstrated in isolated guinea pig ventricular myocytes with comparable time course (~15 min) (Wang et al., 2007). The present results clearly show that the rate of depolarisation was severely reduced in hypoxia, as has been observed in rabbit atrioventricular node (Nishimura, Tanaka, Homma, Matsuzawa, & Watanabe, 1989), and the extent and rate of Na^+ entry were progressively decreased (Fig. 4b). These changes would have direct effects on the exAP amplitude and duration. The reduction in exAP duration, and probably also the amplitude, was likely the result of K_{ATP} channel activation (Shaw & Rudy, 1997; Qi, Shi, Wang, Zhang, & Xu, 2000; Reffelmann, Skobel, Kammermeier, Hanrath, & Schwarz, 2001; Wu, Hayashi, Lin, & Chen, 2005), such as the sarcolemmal K_{ATP} channel (Weyermann, Vollert, Busch, Bleich, & Gogelein, 2004; Chen, Zhu, Wilson, & Cameron, 2005).

(3) The propagation velocity, which is a reflection of the conductivity and contractility of the cultured cardiac myocytes, reduced following hypoxic treatment. It has been suggested that hypoxia could impair atrioventricular nodal conduction by reducing the slow inward current (I_{si}) and the delayed rectifier K^+ (K_{dr}) current (Nishimura et al., 1989). The suppression of the I_{si} may be the result of impaired phosphorylation due to reduced ATP levels (Nishimura et al., 1989). It is known that the K_{dr} channels regulate electrical activity of the cells by providing outward K^+ currents, thus influencing the resting potential (Ishikawa, Eckman, & Keef, 1997). Indeed, both intracellular K^+ and resting membrane potential have been shown to decrease under hypoxia (Nakaya, Kimura, & Kanno, 1985). Since hypoxia reduces the slow component (I_{KS}) of the K_{dr} channels (Hool, 2004), which are involved in the overall repolarisation during the plateau phase of action potential (Carmeliet, 1993), the propagation velocity would thus be reduced as a result.

The present study using the MEA as an experimental platform confirmed that inflicting hypoxia on cardiac myocytes would cause characteristic changes in beat frequency, exAP amplitude, exAP duration, and propagation velocity. These changes are in good agreement with what is generally known about the electrophysiological changes that would occur with cells in hypoxic conditions (Nakaya et al., 1985; Shaw & Rudy, 1997; Wang et al., 2007).

The present MEA system is clearly applicable to research cardiac functions. The detected exAPs and the associated changes demonstrated in this study allow a partial reconstruction of the shape and time course of the underlying physiological and pathophysiological changes of the cells as a result of hypoxia. One major drawback observed in this study is the potentially large variations, as reflected by the large standard errors of the electrophysiological measurements obtained, because the 'absolute' number of cells cannot be controlled. The number of cells seeded on each MEA would directly affect the time it takes them to become physiologically compromised; therefore, the resultant physiological changes would be different over the course of hypoxic insult. Nonetheless, the thus obtained measurements from different chips would still provide the same 'pattern' of change over time. Furthermore, parallel biochemical and molecular analyses of the treated cells could verify and substantiate the significance of the extracellular recordings obtained using the MEA.

5. Conclusions

The present cardiac myocyte-integrated MEA experimental platform is suitable for monitoring the electrophysiological functions of metabolically-compromised cells over a relatively long period of time. This system could be useful for the pharmacological studies of a variety of heart problems. The measured extracellular electrophysiological parameters can provide valuable information on cellular functions, and this system can be used in conjunction with biochemical and molecular techniques in order to provide a comprehensive picture of cellular behaviour in different pathophysiological conditions.

Acknowledgments

This work is supported by an Earmarked Grant from the Research Grant Council of Hong Kong under the contract number 611205 and Helmholtz Association of National Research Centres, Germany. The authors would like to thank N. Wolters (IBN-2, Electronic Workshop) and Y. Zhang (IBN-2) for their technical expertise in designing the current MEA system.

References

Barry, W. H. (2006). Na "Fuzzy space": Does it exist, and is it important in ischemic injury? *Journal of Cardiovascular Electrophysiology*, *17*(Suppl 1), S43−S46.

Belardinelli, L., Shryock, J. C., & Fraser, H. (2006). Inhibition of the late sodium current as a potential cardioprotective principle: Effects of the late sodium current inhibitor ranolazine. *Heart*, *92*(Suppl 4), iv6−iv14.

Bollensdorff, C., Knopp, A., Biskup, C., Zimmer, T., & Benndorf, K. (2004). Na(+) current through KATP channels: Consequences for Na(+) and K(+) fluxes during early myocardial ischemia. *American Journal of Physiology. Heart and Circulatory Physiology*, *286*, H283−H295.

Carmeliet, E. (1993). K+ channels and control of ventricular repolarization in the heart. *Fundamental & Clinical Pharmacology*, *7*, 19−28.

Chen, J., Zhu, J. X., Wilson, I., & Cameron, J. S. (2005). Cardioprotective effects of K ATP channel activation during hypoxia in goldfish *Carassius auratus*. *Journal of Experimental Biology*, *208*, 2765−2772.

Chlopcikova, S., Psotova, J., & Miketova, P. (2001). Neonatal rat cardiomyocytes — A model for the study of morphological, biochemical and electrophysiological characteristics of the heart. *Biomedical Papers of the Medical Faculty of PalackyÂ University in Olomouc, Czech*, *145*, 49−55.

Diaz, R. J., & Wilson, G. J. (2006). Studying ischemic preconditioning in isolated cardiomyocyte models. *Cardiovascular Research*, *70*, 286−296.

Ecken, H., Ingebrandt, S., Krause, M., Richter, D., Hara, M., & Offenhäusser, A. (2003). 64-Channel extended gate electrode arrays for extracellular signal recording. *Electrochimica Acta*, *48*, 3355−3362.

Eigel, B. N., Gursahani, H., & Hadley, R. W. (2004). Na+/Ca2+ exchanger plays a key role in inducing apoptosis after hypoxia in cultured guinea pig ventricular myocytes. *American Journal of Physiology. Heart and Circulatory Physiology*, *287*, H1466−H1475.

Halestrap, A. P., Clarke, S. J., & Javadov, S. A. (2004). Mitochondrial permeability transition pore opening during myocardial reperfusion — A target for cardioprotection. *Cardiovascular Research*, *61*, 372−385.

Halestrap, A. P., Clarke, S. J., & Khaliulin, I. (2007). The role of mitochondria in protection of the heart by preconditioning. *Biochimica et Biophysica Acta*, *1767*, 1007−1031.

Halestrap, A. P., Kerr, P. M., Javadov, S., & Woodfield, K. Y. (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochimica et Biophysica Acta*, *1366*, 79−94.

Hausenloy, D. J., Yellon, D. M., Mani-Babu, S., & Duchen, M. R. (2004). Preconditioning protects by inhibiting the mitochondrial permeability transition. *American Journal of Physiology. Heart and Circulatory Physiology*, *287*, H841−H849.

Hool, L. C. (2004). Differential regulation of the slow and rapid components of guinea-pig cardiac delayed rectifier K+ channels by hypoxia. *Journal of Physiology*, *554*, 743−754.

Ingebrandt, S., Yeung, C. K., Krause, M., & Offenhausser, A. (2001). Cardiomyocyte-transistor-hybrids for sensor application. *Biosensors & Bioelectronics*, *16*, 565−570.

Ingebrandt, S., Yeung, C. K., Staab, W., Zetterer, T., & Offenhausser, A. (2003). Backside contacted field effect transistor array for extracellular signal recording. *Biosensors & Bioelectronics*, *18*, 429−435.

Ishikawa, T., Eckman, D. M., & Keef, K. D. (1997). Characterization of delayed rectifier K+ currents in rabbit coronary artery cells near resting membrane potential. *Canadian Journal of Physiology and Pharmacology*, *75*, 1116−1122.

Ju, Y. K., Saint, D. A., & Gage, P. W. (1996). Hypoxia increases persistent sodium current in rat ventricular myocytes. *Journal of Physiology*, *497*(Pt 2), 337−347.

Krause, M. (2000). *Untersuchungen zur Zell-Transistor Kopplung mittels der Voltage-Clamp Technik*. Germany: Johannes Gutenberg Universtity Mainz.

Krause, M., Ingebrandt, S., Richter, D., Denyer, M., Scholl, M., Sprossler, C., et al. (2000). Extended gate electrode arrays for extracellular signal recordings. *Sensors and Actuators B: Chemical*, *70*, 101−107.

Liu, H., Chen, H., Yang, X., & Cheng, J. (2001). ATP sensitive K+ channel may be involved in the protective effects of preconditioning in isolated guinea pig cardiomyocytes. *Chinese Medical Journal (Engl)*, *114*, 178−182.

Nakaya, H., Kimura, S., & Kanno, M. (1985). Intracellular K+ and Na+ activities under hypoxia, acidosis, and no glucose in dog hearts. *American Journal of Physiology*, *249*, H1078−H1085.

Nishimura, M., Tanaka, H., Homma, N., Matsuzawa, T., & Watanabe, Y. (1989). Ionic mechanisms of the depression of automaticity and conduction in the rabbit atrioventricular node caused by hypoxia or metabolic inhibition and protective action of glucose and valine. *American Journal of Cardiology*, *64*, 24J−28J.

Orita, H., Fukasawa, M., Uchino, H., Uchida, T., Shiono, S., & Washio, M. (1995). Hypoxic injury of immature cardiac myocytes under various hypothermic conditions using an in vitro cell-culture model. *Japanese Circulation Journal*, *59*, 347−353.

Qi, X. Y., Shi, W. B., Wang, H. H., Zhang, Z. X., & Xu, Y. Q. (2000). A study on the electrophysiological heterogeneity of rabbit ventricular myocytes the effect of ischemia on action potentials and potassium currents. *Sheng Li Xue Bao*, *52*, 360−364.

Rajs, J., & Harm, T. (1980). Isolated rat cardiac myocytes as an experimental tool in the study of anoxic cell injury. Effect of reoxygenation — A preliminary report. *Forensic Science International*, *16*, 185−190.

Reffelmann, T., Skobel, E. C., Kammermeier, H., Hanrath, P., & Schwarz, E. R. (2001). Activation of ATP-sensitive potassium channels in hypoxic cardiac failure is not mediated by adenosine-1 receptors in the isolated rat heart. *Journal of Cardiovascular Pharmacology*, *6*, 189−200.

Reppel, M., Pillekamp, F., Lu, Z. J., Halbach, M., Brockmeier, K., Fleischmann, B. K., et al. (2004). Microelectrode arrays: A new tool to measure embryonic heart activity. *Journal of Electrocardiology*, *37*, 104−109 (Suppl).

Saint, D. A. (2006). The role of the persistent Na(+) current during cardiac ischemia and hypoxia. *Journal of Cardiovascular Electrophysiology*, *17*(Suppl 1), S96−S103.

Saint, D. A. (2008). The cardiac persistent sodium current: An appealing therapeutic target? *British Journal of Pharmacology*, *153*, 1133−1142.

Sedmera, D., Kucera, P., & Raddatz, E. (2002). Developmental changes in cardiac recovery from anoxia-reoxygenation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *283*, R379−R388.

Shaw, R. M., & Rudy, Y. (1997). Electrophysiologic effects of acute myocardial ischemia: A theoretical study of altered cell excitability and action potential duration. *Cardiovascular Research*, *35*, 256−272.

Suleiman, M. S., Halestrap, A. P., & Griffiths, E. J. (2001). Mitochondria: A target for myocardial protection. *Pharmacology & Therapeutics*, *89*, 29−46.

Wang, W., Ma, J., Zhang, P., & Luo, A. (2007). Redox reaction modulates transient and persistent sodium current during hypoxia in guinea pig ventricular myocytes. *Pflugers Archiv*, *454*, 461−475.

Webster, K. A., Discher, D. J., & Bishopric, N. H. (1995). Cardioprotection in an in vitro model of hypoxic preconditioning. *Journal of Molecular and Cellular Cardiology*, *27*, 453−458.

Weyermann, A., Vollert, H., Busch, A. E., Bleich, M., & Gogelein, H. (2004). Inhibitors of ATP-sensitive potassium channels in guinea pig isolated ischemic hearts. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *369*, 374−381.

Wrobel, G., Zhang, Y., Krause, H. J., Wolters, N., Sommerhage, F., Offenhausser, A., et al. (2007). Influence of the first amplifier stage in MEA systems on extracellular signal shapes. *Biosensors & Bioelectronics*, *22*, 1092−1096.

Wu, S., Hayashi, H., Lin, S. F., & Chen, P. S. (2005). Action potential duration and QT interval during pinacidil infusion in isolated rabbit hearts. *Journal of Cardiovascular Electrophysiology*, *16*, 872−878.

Yeung, C. K., Ingebrandt, S., Krause, M., Offenhausser, A., & Knoll, W. (2001). Validation of the use of field effect transistors for extracellular signal recording in pharmacological bioassays. *Journal of Pharmacological and Toxicological Methods*, *45*, 207−214.

Yeung, C. K., Sommerhage, F., Wrobel, G., Offenhausser, A., Chan, M., & Ingebrandt, S. (2007). Drug profiling using planar microelectrode arrays. *Analytical and Bioanalytical Chemistry*, *387*, 2673−2680.

Zhang, Y., Wrobel, G., Wolters, N., Ingebrandt, S., Krause, H., Otto, R. (2005). Device for the non-invasive measurement of cell signals. Kernforschungsanlage Juelich, assignee.