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Original Article

Cardioprotective Effects of Potassium Channel Openers on Rat Atria and Isolated Hearts under Acute Hypoxia

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Abstract

Under hypoxia, sarcolemmal K_{ATP} (sarcK_{ATP}) and mitochondrial K_{ATP} (mitoK_{ATP}) channels exert cardioprotective effects via different mechanisms. In the present study, effects of potassium channel openers (KCOs) on electrophysiology (extracellular field potential, exFP) of the hypoxic heart were measured. During re-oxygenation, the mitoK_{ATP} channel opener diazoxide (DZX) reduced the hypoxic effect on the electrophysiology of atria without affecting the exFP duration. DZX also reduced the infarct size of hypoxic hearts (3.01 ± 1.62 %, n = 3). The myocardial cell area changes following KCOs pre-treatment further suggest that DZX-induced cardioprotection in isolated hearts was due to a preservation of cell volume via mitoK_{ATP} channel activation. On the other hand, the sarcK_{ATP} channel opener pinacidil did not show any significant cardioprotective effect on the heart following hypoxia. The present study demonstrates that the activation of mitoK_{ATP} channels may also be involved in cardioprotection following acute hypoxia.

Key words: K_{ATP} channel openers, hypoxia, atria, isolated hearts, electrophysiology, infarct size

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Introduction

Cardiac potassium channels (K^+ channels) play a significant role in controlling heart rate, and different cardioprotective signalling pathways converge on cardiac K^+ channels to modulate the shape and duration of the action potential as well as the resting membrane potential (Tamargo et al., 2004). Amongst all cardiac K^+ channels, ligand-gated adenosine triphosphate-sensitive (K_{ATP}) channels are known to play an active cardioprotective role in ischaemia/hypoxia (Wang et al., 2005). Both sarcolemmal K_{ATP} ($sarcK_{ATP}$) channels, which are located on the cell membrane; and mitochondrial K_{ATP} ($mitoK_{ATP}$) channels, which are expressed in mitochondria, have been demonstrated to be involved in protecting the ischaemic/hypoxic hearts via different mechanisms (Gross et al., 1999). The opening of $sarcK_{ATP}$ channels affects the electrical excitability of cardiomyocytes and reduces energy requirements via shortening of action potentials during acute hypoxia, while the opening of $mitoK_{ATP}$ channels mainly involves gene expression pathways that converge on mitochondria during chronic hypoxia (Gross et al., 1999). The shortening of the action potential duration via the opening of $sarK_{ATP}$ channels has been suggested as a key factor in the observed cardioprotective effects. However, a latter study has suggested that intracellular $mitoK_{ATP}$ channels, which are involved in the control of mitochondrial matrix volume, may also contribute to acute ischaemia/hypoxia (Gross et al., 1999).

The microelectrode array (MEA) has been developed as a non-invasive tool for studying the electrophysiology of cultured cells (Stett et al., 2003). There is, however, relatively little progress being made in studying the electrophysiology of heart slices because there isn't a standardised technique in obtaining intact and viable slices. To date, electrogenically active ventricular slices of embryonic, neonatal, or adult mouse hearts have been isolated and studied using the MEA (Halbach et al., 2006; Pillekamp et al., 2005). The extracellular field potentials (exFPs) of heart slices on MEA chips can be measured; and the changes in frequency, amplitude, and propagation speed can then be calculated (Halbach et al., 2006). The MEA can therefore be used as a tool for studying the effects of pharmacological agents on electrophysiological parameters of the heart. In addition to ventricular slices, atrial slices can also be used as a study model in cardiac research. The sinoatrial node (SA node), which is located in the right atrium, is responsible for generating and propagating electrical activity uniformly over the atrium initially and then to the rest of the heart. It may therefore be equally useful to monitor the exFPs originated from atria using the MEA.

In the present study, the effects of KCOs on the atrium during hypoxia and reoxygenation were studied using the MEA. Changes in exFP rate and exFP duration of the hypoxic atrium and KCOs pre-treated hypoxic atrium were studied. We aimed to investigate whether the change in field potential duration was the sole explanation for the cardioprotective effects of KCOs. Determinations of infarct size, which is the gold standard in revealing the damage present in the heart; and myocardial cell area, which further provides invaluable histological information following different experimental treatments, were also performed on perfused hypoxic hearts. The present study intended to illustrate the protective effect of KCOs on the heart.

Materials and Methods

Solutions and drugs

The standard Krebs-Henseleit solution (pH 7.4) contained (mM): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), $CaCl_2$ (1.25), and D-glucose (10.6).

In this study, K_{ATP} channel openers, pinacidil (PIN, Leo Pharmaceuticals, Denmark) and diazoxide (DZX, Sigma, MO, USA), were used to pre-treat the heart tissues before being subjected to hypoxia. The stock of PIN (10 mM) was made using 40 % (v/v) ethanol in distilled water, while the stock of DZX (10 mM) was made using distilled water (adjusted to pH 8.0 using a small amount of sodium hydroxide). The working concentrations of all K_{ATP} channel openers were made using Krebs' solution (pH 7.4).

Animals

Sprague-Dawley rats were outbred within the Laboratory Animal Services Centre of the Chinese University of Hong Kong and were housed at

approximately 25 °C in 12-h light/dark cycles. The Animal Experimentation Ethics Committee, the Chinese University of Hong Kong, approved the experiments and protocols used.

Studies on the rat atrium using the MEA

Preparation of the rat atrium

Male Sprague-Dawley rats (250 − 300 g) were killed by cervical dislocation. The hearts were removed rapidly and the right atrium from the isolated heart was separated. Atria were then transferred and fixed onto a 3-D MEA chip surface (Ayanda Biosystems, Switzerland) with a tissue holder anchor (Multi Channel Systems, Germany). The 3-D MEA chip has 60 tipped platinum electrodes with an electrode height between 25 and 35 μm and an inter-electrode spacing of 100 μm. The electrode arrays were mounted onto a printed circuit board and then fitted into the MEA60 system interface (Multi Channel Systems, Germany). Data were sampled at 10 kHz per channel with simultaneous data acquisition using MEA60 System (Multi Channel Systems, Germany).

Experimental protocol

After 15 min of stabilisation in continuously oxygenated Krebs' solution at 37 °C, a baseline recording was obtained. Atria were either superfused with normal Krebs' solution or one containing a particular drug for 15 min, followed by a 10 min of hypoxia (Krebs' solution gassed with 95 % N_2/5 % CO_2 gas mixture). The atria were then superfused with the oxygenated Krebs' solution and recordings were taken every 5 min for up to 40 min. The hearts were separated into four groups according to the designated pre-treatments with different drug-containing Krebs' solution before being subjected to hypoxia. A schematic overview of the experimental protocol is shown in Fig. 1a.

Experimental groups were assigned as follows:
Group 1: Normoxia
Group 2: Hypoxia without any pre-treatment
Group 3: PIN (1 μM) pre-treatment + Hypoxia
Group 4: DZX (1 μM) pre-treatment + Hypoxia

All the data were analysed using MC Rack software (Multi Channel Systems MCS GmbH, Reutlingen, Germany). The exFP frequency (spike per minute, spm) was determined and the means of the parameter from all available channels of each MEA chip (i.e. 1 experiment) were calculated before obtaining the final means of all the chips from the corresponding treatment group.

Studies on the isolated rat heart using Langendorff apparatus

Preparation of the isolated heart

Isolated rat hearts were prepared as previously described (Watanabe *et al.*, 2004). Briefly, male Sprague-Dawley rats (250 − 300 g) were killed by cervical dislocation. The hearts were removed rapidly and placed in ice-cold Krebs' solution. The hearts were then mounted on a Langendorff apparatus and perfused retrogradely with Krebs' solution at a constant flow rate of 10 ml/min without recirculation. The pre-warmed Krebs' solution (37 °C) was gassed continuously with 95 % O_2/5 % CO_2 gas mixture. A coronary perfusion pressure of 30 to 40 mmHg was achieved at the beginning of each experiment.

Experimental protocol

After 15 min of stabilisation in continuously oxygenated Krebs' solution at 37 °C, the hearts were either perfused with normal Krebs' solution or one containing a test drug for 15 min, then followed by a 15 min of hypoxia (Krebs' solution gassed with 95 % N_2/5 % CO_2 gas mixture) treatment. The hearts were then reperfused with the oxygenated Krebs' solution for 120 min. The hearts were separated into four groups according to the pre-treatments with different drug-containing Krebs' solution before being exposed to hypoxia. A schematic overview of the experimental protocol is shown in Fig. 1b.

Experimental groups were assigned as follows:
Group 1: Normoxia
Group 2: Hypoxia without any pre-treatment
Group 3: PIN (100 μM) pre-treatment + Hypoxia
Group 4: DZX (100 μM) pre-treatment + Hypoxia

Measurements on infarct size of the heart

After each experiment, the isolated heart was removed and cut into 0.2 cm thick slices. These heart slices were then incubated in 1% 2,3,5-Triphenyltetrazolium chloride (TTC, Sigma) for 20 min at 37°C. The surviving area would be stained red, while the infarct area would appear pale pink. The tissues were then fixed in 10 % formalin to further increase the colour differential between the surviving and infarct areas of the heart. Infarct areas were then traced and measured using ImageJ software (NIH, USA). The percentage of infarct area within each slice was calculated.

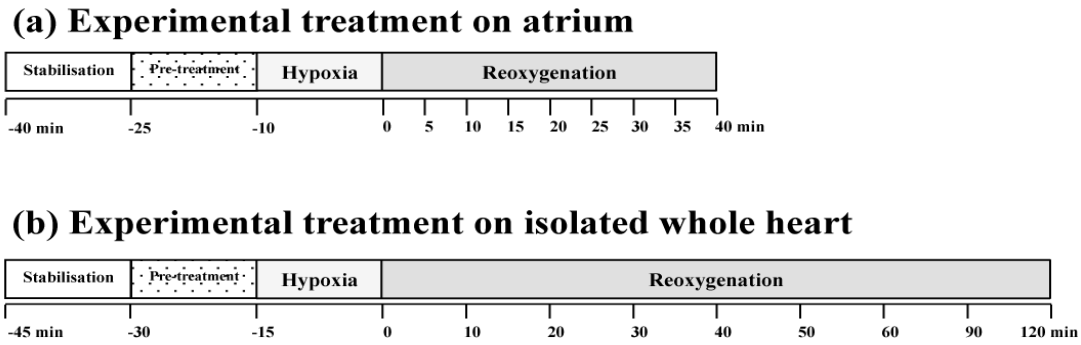

Fig. 1: Protocols of atria experiments (a) and isolated whole hearts (b). (a) After 15 min of stabilisation, the atria were superfused with either normal Krebs' solution or drugs-containing Krebs' solution for another 15 min. The atria then underwent hypoxia for 10 min, followed by reoxygenation for 40 min. The atria were divided into six groups according to different pre-treatments received (n = 4 − 5). (b) After 15 min of stabilisation, the isolated hearts were perfused with either normal Krebs' solution or a drug-containing Krebs' solution for another 15 min. The hearts then underwent hypoxia for 15 min, followed by reoxygenation for 120 min. The hearts were divided into eight groups according to different pre-treatments received (n = 3 − 7).

Measurements on cell area of the myocardium

After each experiment, the atrium was fixed in 4% paraformaldehyde for 24 h, and then later embedded in paraffin wax for slicing and staining. Slices were cut transversely at 5 μm and stained with Hematoxylin-Eosin (H&E). The cell structure was traced along the cell membrane and the cell area was measured using ImageJ software (NIH, USA).

Statistical Analysis

The differences between KCOs pre-treated and untreated atria or isolated hearts under normoxic or hypoxic conditions were analysed using unpaired *t*-test. Two-way ANOVA followed by the Bonferroni post-hoc test was used when the analysis was dependent of time. $P < 0.05$ indicates a significant difference between values. All data are indicated as mean ± S.E.M.

Results and Discussion

Using rat atria and isolated hearts, the electrophysiological effect of PIN and DZX and their cardioprotective property in acute hypoxia were investigated in the present study.

Effects of KCOs on the electrophysiology of rat atria

The atria had a mean control exFP frequency of 175.01 ± 6.74 spm (n = 25). The atria, which were superfused with oxygenated Krebs' solution, maintained a stable exFP frequency throughout the experiment (n = 4) (Fig. 2a). After superfusing the atria with hypoxic Krebs' solution for 10 min, a 33.14 ± 10.27 % (n = 5) reduction in exFP frequency was observed when compared with its control (Fig. 2a). The exFP frequency then progressively increased and was significantly elevated after 40 min of reperfusion with oxygenated solution ($P < 0.05$).

When compared with the untreated atria, the PIN pre-treated atria had a smaller reduction in exFP frequency of only 15.11 ± 9.95 % (n = 4) following hypoxia treatment (Fig. 2b). Changes in the exFP frequency of both untreated and PIN pre-treated atria remained similar, but higher than their

respective controls, during the reoxygenation period. Compared with the untreated atria, atria pre-treated with DZX had a similar reduction in exFP frequency (28.77 ± 10.52 %, n = 4) following hypoxia. The exFP frequency of the DZX pre-treated atria returned to its basal values after hypoxia without any observable elevation (Fig. 2b). Significant differences in exFP frequencies between the untreated and DZX pre-treated atria were found at the latter stage of reoxygenation (t = 35+ min, $P < 0.05$).

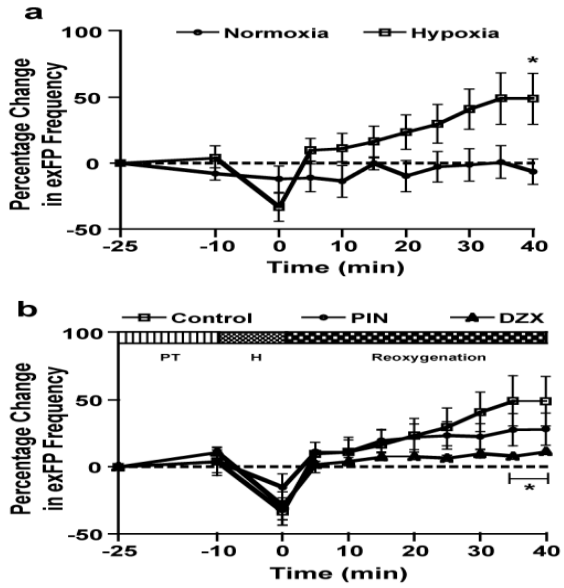

Fig. 2: Changes in extracellular field potential (exFP) frequency (spm) ± S.E.M. of atria, with or without pre-treatments, at different time points (-10, 0, 5, 10, 15, 20, 25, 30, 35, and 40 min). (a) Under normoxic conditions, the exFP frequency remained stable throughout the experiment (n = 4). The exFP rate was slightly reduced after 10 min of hypoxia but then gradually returned to its basal value once the atria were again superfused with oxygenated Krebs' solution (n = 5). The exFP frequencies were elevated progressively and were significantly different from the normoxic atria (t = 40 min, $*P < 0.05$). (b) Atria pre-treated with pinacidil (PIN) had a smaller reduction in exFP frequency after hypoxia than the untreated atria and resumed similar exFP frequency as the untreated atria during reoxygenation (n = 4). Atria pre-treated with diazoxide (DZX) had the same extent of reduction in exFP frequency after hypoxia when compared with the untreated atria. The exFP frequency then returned to its basal frequency after 5 min of reoxygenation (n = 4). Significant differences in exFP frequencies were observed between the untreated and DZX pre-treated atria after 35+ min of reoxygenation ($*P < 0.05$). PT: pre-treatment; H: Hypoxia. The percentage change in exFP frequency is expressed as mean ± S.E.M.

Individual exFP signals were lengthened after hypoxia (Fig. 3a). Compared with the original exFP shape, the exFP of atria pre-treated with PIN or DZX did not cause any distortion or lengthening of their respective signal shapes (Fig. 3b and Fig 3c).

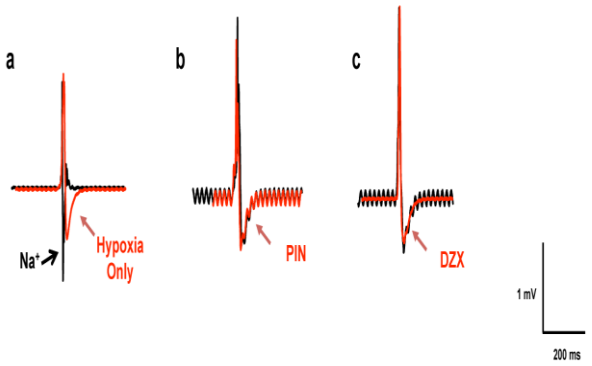

Fig. 3: Representative traces showing individual extracellular field potential (exFP) of atria. (a) The exFP duration was lengthened after being subjected to 10 min of hypoxia (lower panel). The magnitude of the sodium signal reduced and the duration lengthened, indicating the electrochemical gradient of sodium was reduced due to hypoxia. (b) The exFP duration and amplitude were unchanged in the presence of pinacidil (PIN). (c) The exFP duration and amplitude were unchanged in the presence of diazoxide (DZX).

Effects of KCOs on the infarct size of the hypoxic hearts

Hypoxic hearts without undergoing KCOs pre-treatment had an infarct size of 29.24 ± 12.11 % (n = 3) following 120 min of reoxygenation (Fig. 4). Hypoxic hearts pre-treated with either PIN or DZX had a lesser extent of tissue damage than the untreated hearts, with infarct size measurements of 15.22 ± 5.45 % (n = 3) and 3.01 ± 1.62 % (n = 3, $P < 0.05$), respectively (Fig. 4).

Effects of KCOs on the cell area of the rat myocardium

Rat myocardium after being exposed to different experimental conditions was stained with H&E (Fig. 5a). The cell area of the myocardium increased from 177.99 ± 8.74 μm^2 (n = 3) in normoxic hearts to 254.19 ± 34.48 μm^2 (n = 3, $P < 0.05$) in hypoxic hearts. Following PIN pre-treatment, the cell area was further increased to 351.04 ± 73.46 μm^2 (n = 3). On the other hand, DZX pre-treatment reduced the cell area to 185.65 ± 27.80 μm^2 (n = 3) (Fig. 5b).

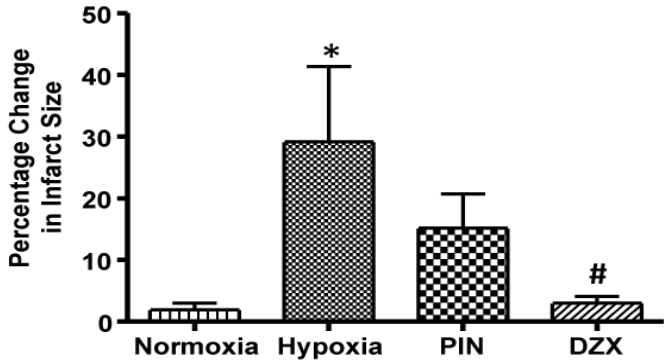

Fig. 4: Changes in infarct size (%), with or without pre-treatments, after being exposed to 15 min of hypoxia and 120 min of reoxygenation. Hypoxia increased infarct size of the hearts (*P < 0.05). Reduction in infarct size was observed when the hearts were pre-treated with pinacidil (PIN) or diazoxide (DZX, $^{\#}P$ < 0.05) before being subjected to hypoxia. The percentages of infarct size of the hearts are expressed as mean ± S.E.M, n = 3.

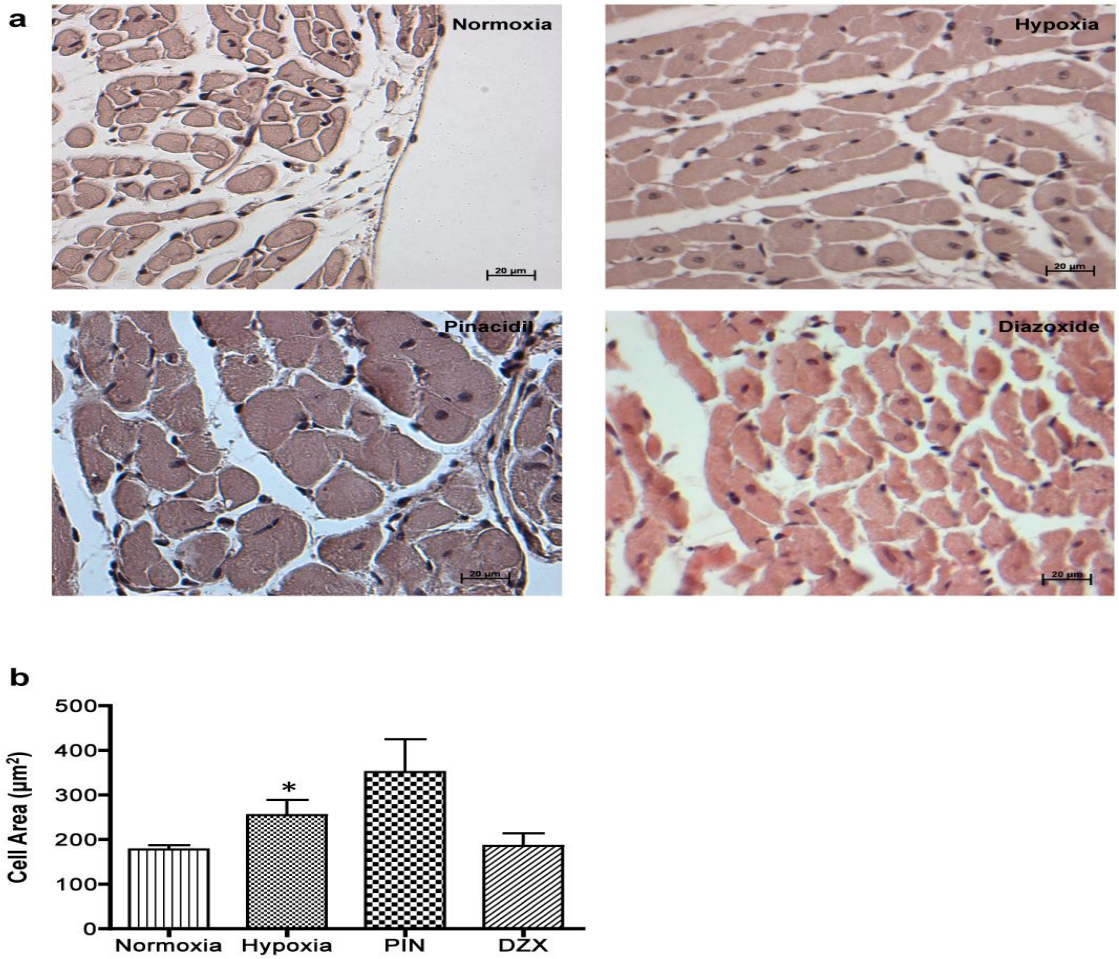

Fig. 5: The effects of potassium channel openers on myocardial cell area (µm^2 ± S.E.M). (a) Representative H&E staining of myocardium after different experimental conditions. The cell body is stained red, while the nucleus is stained dark blue. (b) Hypoxic cell area was increased (*P < 0.05) probably due to the accumulation of lactate during hypoxia, and the size of the cells was further increased in the presence of pinacidil (PIN). Diazoxide (DZX) slightly reduced the cell swelling effect when compared with the untreated hypoxic cells. Results are expressed as mean ± S.E.M, n = 3.

Previous studies have demonstrated that sarcK_{ATP} channels are the most prominent targets of cardioprotection by shortening the cardiac action potential and by suppressing reperfusion-triggered arrhythmia (Wang *et al.*, 2005; Wilde *et al.*, 1994). Recent evidence has shown that mitoK_{ATP} channels may also exhibit cardioprotective effects by regulating mitochondria in acute hypoxia (Das *et al.*, 2003). In the present study, the effects of sarcK_{ATP} and mitoK_{ATP} channel openers on the electrophysiology of atria and on the histology of isolated hearts under acute hypoxic conditions were studied.

Compared to dissociated cardiac cells, heart slices or sections of the heart with a preserved tissue structure serve as a better model for electrophysiological and histological studies in cardiac research (Bussek *et al.*, 2009). The autorhythmic characteristic of the SA node serves as a suitable target for studying the electrophysiological changes of the heart in the presence of different pharmacological agents. In addition, several clinical studies have demonstrated that human right atrial tissues from patients are sensitive to KCO-induced preconditioning (Cleveland *et al.*, 1997; Hanouz *et al.*, 2002; Riddle, 2003). As such, atrial tissues may also be useful for studying the preconditioning effects of KCOs. In the present study, exFP frequencies and the shape of exFPs of atria that had undergone KCOs pre-treatment were studied using the MEA. The electrophysiology of the atria was sensitive to hypoxia, with observable changes in exFPs frequency (Fig. 2a) and exFP duration (Fig. 3a) following 10 min of hypoxia. The reduction in sinus rate immediately after hypoxia may be the result of a shift in the threshold potential to less negative values due to the accumulation of extracellular K^+ (Benndorf *et al.*, 1997). Amongst all the factors that could potentially increase the automaticity of the heart, hypoxia-induced acidosis might explain the progressive changes in exFP frequency during reoxygenation as observed in the present study.

In the present study, a reduction in exFP changes of the hypoxic atria by the mitoK_{ATP} channel opener DZX, but not the sarcK_{ATP} channel opener PIN, was observed during reoxygenation (Fig. 2b). The exFP shapes of atria were compared before and after pre-incubation with KCOs, and none of them were altered in the present study (Fig. 3). The electrophysiological data suggest that the protective effects observed in the present study are unrelated to the changes in field potential duration.

A study has suggested that the mitoK_{ATP} channels involve matrix volume regulation; and the opening of mitoK_{ATP} channels can lead to membrane depolarisation, a slowing of ATP synthesis, a reduction in Ca^{2+} overload, and cell swelling. The attenuation of Ca^{2+} overload thus serves as a cardioprotective mechanism towards hypoxia/ischaemia (Gross *et al.*, 2003). Further heart damage was assessed by determining infarct size, and the results obtained were in line with those found in physiological studies. Hypoxic hearts pre-treated with DZX had comparable reduced tissue damage than the un-treated hearts (Fig. 4).

Other studies have suggested that the cardioprotective effects of K_{ATP} channels are executed via the regulation of cell volume (Diaz *et al.*, 2003; Shi *et al.*, 2009). The accumulation of intracellular and osmotically active lactate promotes cell swelling and cell death (Wright *et al.*, 1998). Results from KCOs pre-treated hearts after reoxygenation show that the cell area of PIN pre-treated hearts was larger than the untreated hypoxic hearts (Fig. 5b). In order to compensate for PIN-induced K^+ efflux, an equivalent Cl^- efflux is needed, and water enters the cells due to changes in osmolality (Shi *et al.*, 2009). The cell area of DZX pre-treated hearts, on the other hand, was slightly smaller than that found in the untreated hypoxic hearts. The cell volume preservation by the opening of mitoK_{ATP} channels may be one of the explanations for the cardioprotective characteristic observed in the present study.

Conclusion

The present study shows that the mitoK_{ATP} channels may be actively involved, without altering the field potential duration, in the mechanism of cardioprotection following acute hypoxia. The ability to regulate cell volume by activating mitoK_{ATP} channels may be an underlying mechanism of cardioprotection.

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