A Study of the Relationship Between Pharmacologic Preconditioning and Adenosine Triphosphate-Sensitive Potassium (K_{ATP}) Channels on Cultured Cardiomyocytes Using the Microelectrode Array

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Abstract: The microelectrode array was used to study the pharmacologic preconditioning effect of adenosine triphosphate-sensitive channel activation using potassium channel openers (KCOs) on rat cardiomyocytes over 90 minutes of ischemia. Cell viability and electrophysiological changes between KCOs pretreated and untreated cardiomyocytes were compared. Ischemia caused significant increases in beat frequency, extracellular field potential amplitude, and propagation velocity of spontaneously beating untreated cardiomyocytes. However, these electrophysiological parameters reduced as the duration of ischemia increased. The electrophysiological changes on ischemic cardiomyocytes were abolished by pretreating the cells with KCOs. Pinacidil pretreated cardiomyocytes retained a significantly higher viability than the untreated cardiomyocytes after 90 minutes of ischemia. Because Connexin 43 has a direct correlation with the propagation velocity, the Connexin 43 protein expression was also investigated. Connexin 43 expression levels were lower in KCOs pretreated cardiomyocytes than that of the untreated controls, and this correlated with the propagation velocity results obtained from the microelectrode array. The effect of pinacidil (sarcolemmal adenosine triphosphate-sensitive channel opener) was more prominent than that of diazoxide (mitochondrial K adenosine triphosphate-sensitive channel opener) on ischemic cardiomyocytes as indicated in the present acute ischemia study.

Key Words: ischemia, pharmacologic preconditioning, K_{ATP} channels, potassium channel openers, microelectrode array, rat cardiomyocytes

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INTRODUCTION

Ischemic preconditioning (IPC) and pharmacologic preconditioning have been demonstrated to have cardioprotective effects against ischemia.[1] With multiple brief exposures to ischemia or pharmacologic agents that induce preconditioning, subsequent prolonged ischemia causes less damage to the cardiac cells in terms of infarct size and mortality.[2,3] Preconditioning establishes a two-phase cardioprotective mechanism: a transient state that appears immediately and lasts for 2 to 3 hours and a delayed state that appears 12 to 24 hours after the initial preconditioning stimulus and lasts for 3 to 4 days.[4] The transient effect of preconditioning provides a means of cardioprotection during cardiopulmonary bypass surgery. Indeed, myocyte injury is reduced in patients with direct IPC before surgery.[5] However, another study indicated that pharmacologic preconditioning may be more desirable than IPC, because IPC may further endanger patients with diseased myocardial.[6]

The activation of G protein coupled receptors by various G protein coupled receptor agonists, eg, adenosine, opioids, bradykinin, and volatile anesthetics, has been demonstrated to be involved in IPC and pharmacologic preconditioning, which are known to have a cardioprotective ability on ischemic cells.[7–10] These agonists bind to membrane-bound G_i receptors and activate several molecular signaling pathways.[11] The activations of protein kinase C, tyrosine kinase, and mitogen-activated protein kinase pathways are believed to play the most significant role among different cardioprotection-related signaling pathways.[12] It is believed that cardiac potassium channels (K^+ channels) are the end-effector of the preconditioning mechanism because K^+ channels play a major role in controlling the resting membrane potential, the shape and duration of the action potential, and consequently heart rate.[13] Among different types of cardiac K^+ channels, the ligand-gated adenosine triphosphate-sensitive (K_{ATP}) channel is a target for studying the importance of K^+ channels in ischemia/hypoxia.[14] There are two K_{ATP} channel subtypes found in cardiomyocytes: one that is located on the cell membrane, the sarcolemmal K_{ATP} (sarcK_{ATP}) channel, and one that is expressed in mitochondria, the mitochondrial K_{ATP} (mitoK_{ATP}) channel. The underlying cardioprotective mechanisms elicited by the opening of K_{ATP} subtypes are different.[15] The sarcK_{ATP} channel affects the electrical excitability of

cardiomyocytes, whereas the mitoK_{ATP} channel involves gene expression pathways that converge on mitochondria.[15] The participation of different K_{ATP} subtypes at different stages of ischemia remains to be fully elucidated.

Gap junction proteins control the electrical communications in the heart by connecting adjacent cells through arrays of intercellular channels. The resultant synchronized cardiac action potentials between cells coordinate the functionality of the heart.[16] It is believed that changes in gap junction protein expression, distribution, and property may be involved in various cardiac diseases.[17] Connexin-43 (Cx43) is the most abundant gap junction protein expressed in the mammalian heart, and its expression is altered during ischemia.[18] An initial elevation of Cx43 expression is observed immediately after ischemia, and this is followed by a subsequent reduction after a prolonged ischemic period.[19] Several studies have demonstrated that changes in gap junction protein expression affect the electrical conductance of the heart[20,21] and may underlie the high incidence of arrhythmia-associated cardiac pathology.

In the present study, we focused on the cardioprotective effect of K^+ channel openers (KCOs) on rat cardiomyocytes undergoing up to 90 minutes of ischemic treatment induced by placing a layer of oil on top of a cell monolayer or a cell pellet. This ischemia model was based on a methodology developed by Armstrong et al.[22] The microelectrode array (MEA) was used as a real-time detection tool for measuring the electrophysiological changes in cardiomyocytes during normoxia and ischemia, whereas trypan blue viability assay was used as an end-point detection method for verifying the effects of ischemia and preconditioning on the cells. We aimed to further correlate the transient cardioprotective effects of KCOs with Cx43 expression after the ischemic insult.

MATERIALS AND METHODS

Solutions and Drugs

The standard culture medium was Ham's F10 medium (Sigma, St. Louis, MO; N6635) containing 10 % (v/v) fetal bovine serum (Gibco, Carlsbad, CA; 10270-106), 0.5 % (v/v) insulin, transferrin, selenite solution (Sigma; I1884), 6 mM L-glutamine (Sigma; G7513), and a 2% (v/v) mixture of penicillin/streptomycin (Gibco; 15140-122) adjusted to pH 7.4 using sodium hydroxide.

In this study, different KCOs were used: 1) pinacidil (PIN; Leo Pharmaceuticals Ballerup, Denmark); and 2) diazoxide (DZX; Sigma). 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 at pH 8.0 (pH adjusted using a small amount of sodium hydroxide). The working concentration of both KCOs (100 μM) was made using the standard culture medium (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-hour light/dark cycles. The Animal Experimentation Ethics Committee, the Chinese University of Hong Kong, approved the experiments and protocols that were used.

Cell Culture

Hearts of embryonic Day 19 to 20 Sprague-Dawley rats were prepared according to a previously published protocol.[23] Briefly, hearts were removed, minced, and placed into cold Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (Sigma; H6648). These tissue fragments were digested by stepwise 0.25% trypsin with EDTA (Gibco; 25200-056). After 8 minutes incubation with trypsin at 37°C, the supernatant was discarded. The dissociation cycle then began with another 2 mL of trypsin for 8 minutes preceded by the addition of 100 μL DNase II solution (Sigma; D8764) for 1 to 2 minutes. The resulting supernatant was collected and added into the culture medium containing 20 % (v/v) fetal bovine serum to stop trypsin digestion. This dissociation cycle was repeated four to five times. This cell suspension mixture was centrifuged at 2000 rpm for 5 minutes. The resuspended pellet was cultured in standard medium and incubated for 1 hour at 37°C for the purpose of differential adhesion. This procedure allows fibroblasts to adhere to the culture dish preferentially over cardiac myocytes and thus increases the myocyte-to-fibroblast ratio of the cell suspension.

Experimental Protocols of Preconditioning Cycles and Induction of Ischemia

To induce preconditioning, the cardiomyocytes were preincubated in standard medium with or without KCOs for 5 minutes followed by another 5 minutes preincubation in standard medium without KCOs. This preconditioning cycle was repeated three times. During each medium change, a thin layer of medium would be left on top of the entire syncytium to avoid drying, and fresh medium or one that contained KCO would then be added to the MEA and mixed with the remaining medium at this point. Different concentrations of KCOs (1, 10, and 100 μM) were used in our preliminary MEA study (data not shown) with the effect of 100 μM of KCOs on the electrophysiology of ischemic cardiomyocytes being the most significant. Indeed, several preconditioning studies have demonstrated that 100 μM was the optimal concentration.[24–26] As such, this concentration was chosen in the present study. To induce ischemia, cardiomyocytes were covered with a layer of oil (200–1500 μL) depending on the surface area of the substrate.

Experimental groups were assigned as follows: Group 1, normoxia group; Group 2, ischemia group; Group 3, PIN (100 μM) preincubation + ischemia group; and Group 4: DZX (100 μM) preincubation + ischemia group.

Cell Viability

Primary cardiomyocytes were prepared according to the digestion protocol stated previously and the cell viability experiment was performed according to a previously published protocol.[22] The cell suspension (approximately 1 million cells/mL) was equally divided into four experimental groups (Group 1 to 4), and each aliquot of cell suspension was then placed in a 1.5-mL Eppendorf. Groups 3 and 4 were then subjected to the preconditioning cycles. Afterward, Groups 2, 3, and 4 were centrifuged and subsequently covered with 100 μL of standard medium, and then 200 μL of oil (covering a surface area of 0.50 cm^2 with a depth of 0.40 cm) was added

on top of it. At each corresponding time point (0, 30, 60, and 90 minutes) of ischemia, a 10-μL sample of the cell suspension was removed and mixed with 40 μL of counting medium (0.4% trypan blue in buffer).

Detection of Extracellular Electrophysiology

The MEA was used to measure extracellular field potentials (exFPs) as well as other electrophysiological parameters. Isolated cardiomyocytes (approximately 75,000 cells per chip) were seeded on a fibronectin (Sigma; F0635)-primed MEA surface, and they would beat spontaneously after 2 to 4 days in culture. An initial control recording of 30 seconds of cells incubated in 20 μL of standard medium (covering a surface area of 0.38 cm^2 with a depth of 0.52 cm) was taken before adding 200 μL of oil on top. Thereafter, recordings of 30 seconds were taken at 5, 10, 15, 20, 30, 60, and 90 minutes. All four experimental groups were measured using the same recording protocol. The headstage was placed on a thermostatically controlled hot plate set at 37°C to ensure a consistent and optimal temperature throughout the recording period.

Data were sampled at 10 kHz with simultaneous acquisition across all channels (PCI 6071E; National Instruments, Hong Kong, China) using MED64 conductor 3.1 software (Alpha MED Sciences Co Ltd, Ibaraki, Osaka, Japan). The results were subsequently analyzed using a program implemented in MATLAB (Version 7.0, The MathWorks, Natick, MA). The 1) beat frequency (beats per minute); 2) exFP amplitude (from the positive peak of depolarization to the negative peak of the sodium (Na^+) signal, mV); and 3) propagation velocity (a measure of conductivity among the cardiomyocytes, cm/s) were expressed. The means of these parameters from all available channels of each MEA chip were calculated (ie, n = 1) before obtaining the final means of all the chips from the corresponding treatment group. Because the cells on different MEA chips would have different basal beat frequencies, amplitudes, and conduction velocity, the data were normalized against their respective control and expressed as percent change of these parameters ± standard error of mean.

Immunoblotting

Cardiomyocytes were cultured on six-well culture dishes for 3 to 4 days before the experiments were carried out. Cultured cardiomyocytes were subjected to the preconditioning cycles, according to their respective experimental groups, before being covered with a layer of oil (1.5 mL was used to cover a surface area of 8.81 cm^2 with a depth of 0.17 cm). Cultured cardiomyocytes were removed at 0, 5, 15, 30, 60, and 90 minutes for protein assay. Treated cells were lysed in ice-cold lysis buffer (50 mM Trizma base, 100 mM NaCl, 5 mM EDTA, 67 mM sodium pyrophosphate, 0.01% Triton X-100, and 4% 25× complete protease inhibitors) for 1 hour. Samples were then electrophoresed on a 10 % sodium dodccylsulfate–polyacrylamide gel and transferred to nitrocellulose. Prestained molecular weight markers were electrophoresed in parallel. After incubating the sample in 5% nonfat milk (Anlene, Hong Kong, China) for 1 hour at room temperature to block nonspecific binding, the nitrocellulose was probed with primary connexin-43 antibody (Invitrogen, Carlsbad, CA). The anticonnexin-43 antibody was used at a 1:1000 dilution in 5% bovine serum albumin and 0.03% NaN_3 overnight at 4°C. The nitrocellulose was washed three times, 5 minutes each, with Tris buffer saline (50 mM Tris, pH 7.5, and 150 mM NaCl) and incubated with 1:2000 dilution of antirabbit IgG horseradish peroxidase-linked antibody (Cell Signaling) for 2 hours at room temperature. The nitrocellulose was washed three times with Tris buffer saline as described previously before being subjected to chemiluminescent detection using the ECL Western Blotting Detection Reagents (Amersham, Buckinghamshire, UK). Western blotting data were analysed using ImageJ Software (National Institutes of Health, Bethesda, MD).

Statistics

The differences between the KCOs pretreated and KCOs untreated cardiomyocytes under normoxic or ischemic conditions were analyzed using two-way analysis of variance with post hoc Bonferroni *t* tests as appropriate. $P < 0.05$ indicates a significant difference between values.

RESULTS

The present study showed the viability and electrophysiological differences between KCOs (PIN or DZX) pretreated and untreated ischemic cardiomyocytes. The changes in cell electrophysiology were then correlated with the levels of gap junction protein Cx43 expression. Preliminary electrophysiological study on the effect of KCOs on normoxic cells showed that the same concentration (100 μM) used in the later ischemic study was effectively altering the electrophysiology of the normoxic cells during preconditioning (data not shown). The electrophysiology of the KCOs pretreated cells, however, returned to its untreated levels after being incubated in the ordinary medium for 5 minutes before the start of the experiment. The effect of KCOs on normoxic cell viability is insignificant because the electrophysiology of KCOs pretreated cells remains active.

Effect of Potassium Channel Openers on the Viability of the Ischemic Cardiomyocytes

There was an insignificant drop in cell viability of the untreated normoxic cardiomyocytes after 90 minutes with viability remaining between 78.4 ± 3.7% and 74.2 ± 4.7% (n = 4, $P > 0.05$) under normoxic conditions. However, there was a 12% reduction in cell viability of the untreated cardiomyocytes, reducing from 77.6 ± 2.6% to 65.3 ± 4.4% (n = 17, $P < 0.05$) after 90 minutes of ischemia treatment (Fig. 1). Although KCOs pretreated cardiomyocytes became more resistant to the effect of ischemia, PIN pretreated cells fared better with only a 3% reduction in cell viability from 82.7 ± 1.7% to 79.9 ± 3.3% (n = 8); whereas the DZX pretreated cells still exhibited a 11% reduction in viability from 84.3 ± 2.8% to 73.8 ± 4.5% (n = 8) (Fig. 1).

Effect of Ischemia on the Electrophysiology of the Cardiomyocytes

Under normoxic conditions, the cultured cardiomyocytes had a control beat frequency of 20.4 ± 4.2 beats per minute, an exFP amplitude of 1.68 ± 0.33 mV, and

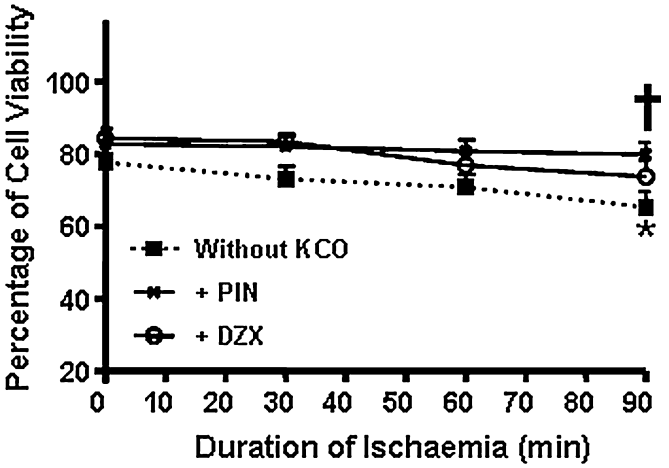

FIGURE 1. Viability of isolated embryonic rat cardiomyocytes under ischemia in the absence or presence of potassium channel openers (KCOs). Cultured cardiomyocytes were pretreated with either pinacidil (PIN;100 μM, n = 8) or diazoxide (DZX; 100 μM, n = 8) before subjecting them to ischemia for 90 minutes. Cell viability at different time points was assessed by the trypan blue exclusion method. There was a 12% reduction in cell viability of the untreated cardiomyocytes, reducing from 77.6 ± 2.6% to 65.3 ± 4.4% (n = 17, $*P < 0.05$) after 90 minutes. A higher viability was generally observed in the presence of KCOs, but significant increases were only observed in PIN pretreated cells at 90 minutes ($\dagger P < 0.05$, with PIN versus without). Cell viability (%) is expressed as mean ± standard error of mean.

a propagation velocity of 14.5 ± 3.6 cm/s (n = 5). The beat frequency of the ischemic cells increased significantly as the duration of ischemia increased (n = 17, $P < 0.01$). Compared with its basal frequency, there was a 66.3 ± 20.2% increase in beat frequency after 5 minutes of ischemia with a peak of 103.0 ± 36.3% at 30 minutes (Fig. 2A). Although the beat frequency declined after 90 minutes of ischemia, it still remained 60.5 ± 46.8% faster than the initial value (Fig. 2A). There was also a significant difference in beat frequencies between the normoxic group (n = 5) and the ischemic group (n = 17) over the first 60 minutes of recording ($P < 0.01$).

The exFP amplitude remained stable over the recording period under normoxic conditions (Fig. 2B). However, there was a 26.5 ± 7.8% increase in exFP amplitude after only 5 minutes of ischemia (Fig. 2B). When compared with the exFP amplitude at baseline, there was a 13.9 ± 18.7% reduction in amplitude after 90 minutes of ischemia.

There was a slight increase in the propagation velocity of cells under normoxic conditions, ranging from 5.7 ± 2.1% to 16.6 ± 8.1% during the 90-minute recording period (Fig. 2C). The propagation velocity of the ischemic group increased from 29.5 ± 11.5% to 42.4 ± 12.4% in the first 20 minutes of ischemia and then reduced to 11.5 ± 24.3% of its baseline after 90 minutes of ischemia (Fig. 2C).

There were no significant changes in the shape of exFP after 90 minutes of recording during normoxia (Fig. 3A). However, the rate of depolarization (fast up-spike) and the extent of Na^+ influx (fast down-spike) were reduced after 90 minutes of ischemia, and the duration of the Na^+ influx signal was also prolonged at the end of the 90-minute period (Fig. 3B).

Effects of Potassium Channel Openers on the Electrophysiology of the Ischemic Cardiomyocytes

The beat frequencies of both KCOs pretreated cardiomyocytes changed gradually over 90 minutes of ischemia. Thus, there was a 50.7 ± 27.7% (n = 7) increase in PIN pretreated cells after 10 minutes of ischemia, whereas the beat frequency of DZX pretreated cardiomyocytes was relatively stable and increased by only 22.5 ± 24.6% (n = 7) even after 20 minutes of ischemia (Fig. 4A). The beat frequencies of both KCOs pretreated cardiomyocytes were then reduced back to their initial values after 60 minutes of ischemia, and in some cases, the beat frequencies even dropped below these values after 90 minutes of ischemia (Fig. 4A). There was a significant difference in beat frequency between the ischemic group and the DZX pretreated groups over the first 60 minutes of ischemia (n = 17; $P < 0.01$).

The exFP amplitudes of PIN or DZX pretreated cardiomyocytes were elevated to 13.6 ± 12.5% (n = 7) and 36.0 ± 10.5% (n = 7), respectively, after being exposed to ischemia for only 5 minutes (Fig. 4B). Thereafter, the exFP amplitudes of PIN or DZX pretreated cardiomyocytes began to decline and dropped below their basal values after 60+ minutes of ischemia (Fig. 4B).

The propagation velocities of PIN or DZX pretreated cardiomyocytes were elevated by 24.1 ± 18.1% (at 10 minutes ischemia, n = 7) and 17.1 ± 15.2% (at 5 minutes ischemia, n = 7), respectively (Fig. 4C). The propagation velocity changes observed in both KCOs pretreated cardiomyocytes reduced to their respective basal values after 30 minutes of ischemia (Fig. 4C). There were no observable changes in the rate of depolarization or the duration of Na^+ influx signal in either PIN or DZX pretreated cells after 90 minutes of ischemia (Fig. 5, insets), but the extent of Na^+ influx signal (fast down-spikes) was reduced in both cases (Fig. 5).

Effects of Potassium Channel Openers on Gap Junction Protein Expression

The untreated cardiomyocytes had a significant increase in Cx43 protein expression at 5 minutes of ischemia (n = 3; $P < 0.05$), but the expression levels decreased gradually over the remaining ischemic treatment period (15–90 minutes) (Fig. 6). When compared with the untreated cardiomyocytes, the Cx43 protein expression levels of PIN pretreated cardiomyocytes were lower over all the ischemic time points measured (Fig. 6B). The Cx43 protein expression levels of DZX pretreated cardiomyocytes were lowered at the first 30 minutes of ischemia but then returned to levels similar to that of the untreated cells (Fig. 6B).

DISCUSSION

The present study demonstrated that both PIN and DZX had beneficial effects, in terms of cell viability and cell

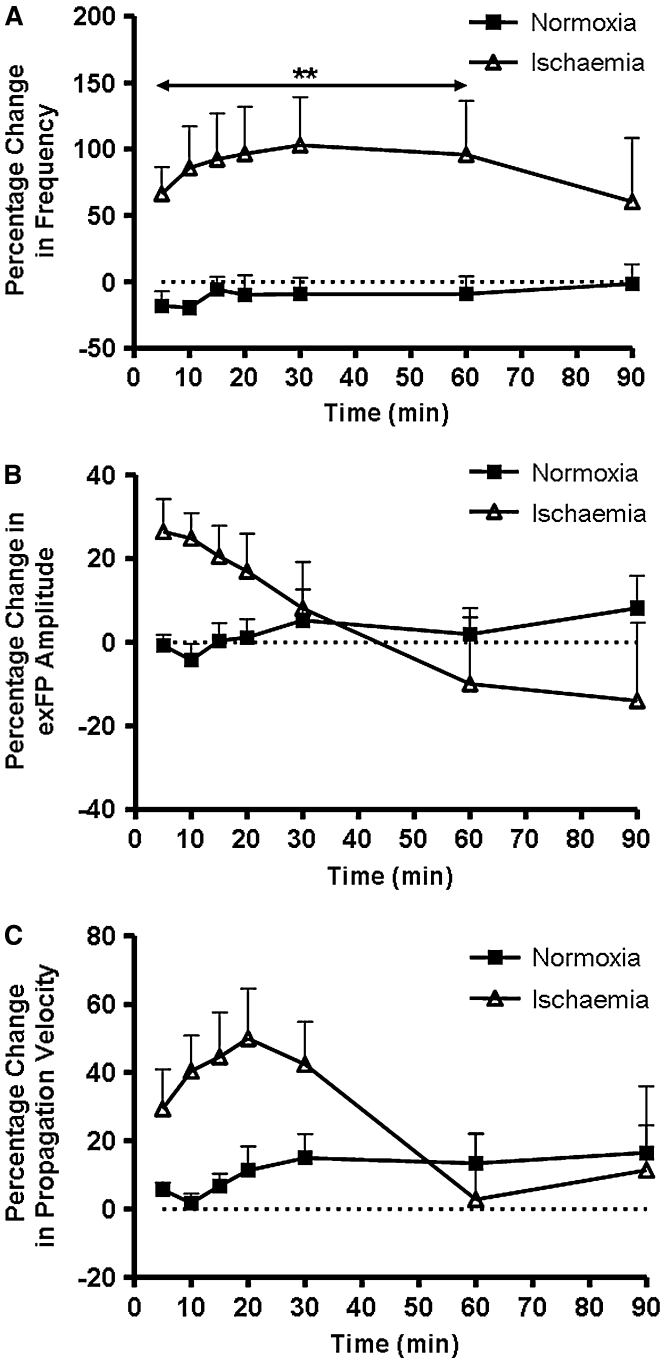

FIGURE 2. Changes in beat frequency (bpm, A), extracellular field potential (exFP) amplitude (mV, V), and velocity (cm/s, C) ± standard error of mean of cultured cardiomyocytes at different time points (5, 10, 15, 20, 30, 60, and 90 minutes) (n =14). (A) The beat frequency elevated immediately after 5 minutes of ischemia and peaked at 30 minutes. The beat frequency then decreased gradually. There were significant changes between cells under normoxic and ischemic conditions (**$P < 0.01$). (B) The exFP amplitude increased as soon as the cells were exposed to ischemia, but this reduced after 10 minutes. At the end of the experiment (90 minutes), the exFP amplitude reduced below the normoxic value (dotted line). (C) The propagation velocity of the cells increased immediately after ischemia and peaked at 20 minutes before reducing after 30+ minutes. Both exFP and propagation velocity reached their respective lows at about 60 to 90 minutes. The percentage change in parameters is expressed as mean ± standard error of mean.

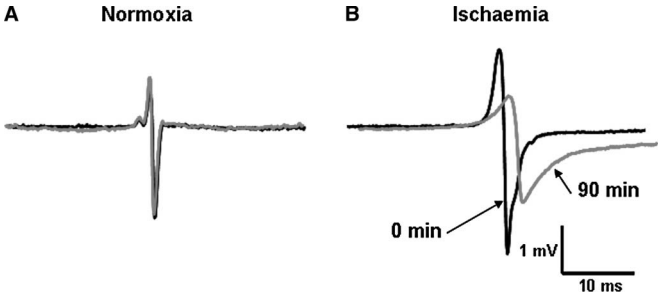

FIGURE 3. Representative traces of individual extracellular field potentials (exFP) of rat cardiomyocytes before and after 90 minutes of normoxic or ischemic treatment. (A) The shape of exFP of the normoxic cells was unchanged in normoxic conditions. (B) After 90 minutes of ischemia, the cells showed observable changes in the rate of depolarization, the rate and the extent of the Na^+ influx, and the duration of the Na^+ influx signal.

electrophysiology, on ischemic cardiomyocytes. When compared with the untreated ischemic cells, gap junction Cx43 protein expression levels were lower in KCOs pretreated cells.

The advantage of using isolated cardiomyocytes as opposed to the whole heart is that other cell types and the nervous system are not present, permitting a simpler interpretation of data. This is important when studying cell-signaling pathways involved in cardioprotective mechanisms.[27] The present study used an ischemic pellet model to mimic ischemic conditions for isolated cardiomyocytes.[22] This ischemic pellet model is useful because it combines severe ischemia with a gradually increasing buildup of metabolites in an enclosed space.[27] There are a number of ways to study the preconditioning effects on isolated ischemic cells with cell viability being one of the most common end-point parameters.[27] By determining the percentage of cardiomyocytes that were stained with trypan blue after resuspension of samples obtained from the ischemic pellet at different time points, the degree of injury with increasing duration of ischemia can be assessed. The reduction in ischemia-induced cell death by exposing cultured cells to KCOs was observed in the present study (Fig. 1).

Although extensive studies have been carried out on cardioprotective effects as a result of activation of $sarcK_{ATP}$ (which PIN activates) and $mitoK_{ATP}$ channels (which DZX activates), the findings thus far are inconclusive.[28,29] The present study demonstrated that both K_{ATP} channels were involved in the preconditioning mechanism of isolated cardiomyocytes and that PIN exerted a better cardioprotective effect than DZX did on ischemia-induced cell death. Because $sarcK_{ATP}$ channels are involved in acute ischemia,[13] this explains why PIN was more effective than DZX in the present acute ischemic system.

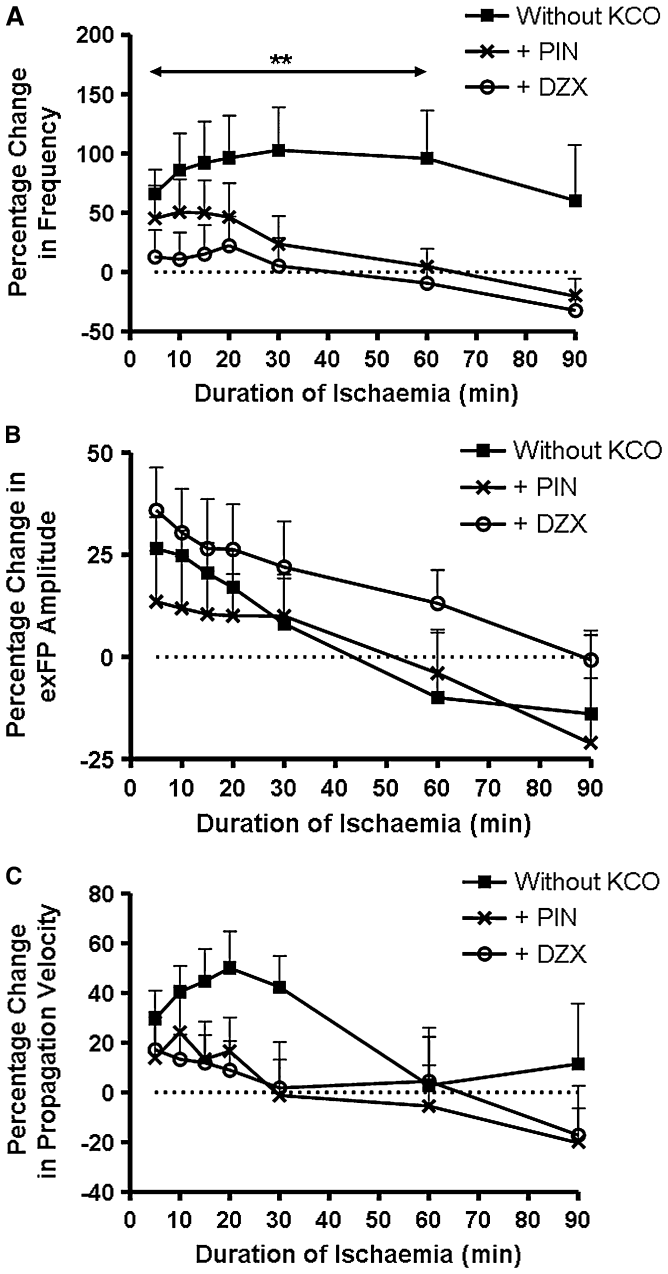

FIGURE 4. Changes in beat frequency (bpm, A), extracellular field potential (exFP) amplitude (mV, B), and velocity (cm/s, C) ± standard error of mean of ischemic rat cardiomyocytes with or without potassium channel openers (KCOs) pretreatment at different time points (5, 10, 15, 20, 30, 60, and 90 minutes). Both pinacidil (PIN) and diazoxide (DZX) had similar effects on ischemic cells. (A) The beat frequencies of KCOs pretreated cells returned to normoxic levels (dotted line) between 30 and 60 minutes, but they dropped below this value by 90 minutes. There were significant changes in the beat frequency between DZX pretreated and untreated cells under ischemic conditions (**$P < 0.01$). (B) The exFP amplitude of both KCOs pretreated cardiomyocytes peaked at 5 minutes and then gradually reduced to normoxic values (dotted line) at 60+ minutes of ischemia. (C) The preconditioning effects by KCOs affected the propagation velocity of the ischemic cells. The velocity of the KCOs pretreated cardiomyocytes returned to normoxic levels (dotted line) at 30+ minutes. The percentage change in parameters is expressed as mean ± standard error of mean.

Changes in cardiac electrophysiology, which were monitored by the MEA in real-time, reflect the condition of an intricate cellular network mimicking the intact heart. The results showed that the beat frequency of cells was initially elevated under ischemic stress followed by a gradual reduction over the remaining duration of ischemia (Fig. 2A). An observable fluctuation in beat frequency of ischemic cells has been reported, and this can subsequently induce tachycardia.[30,31] The exFP amplitude was also initially elevated and then gradually reduced under ischemia (Fig. 2B). The exFP amplitude is composed of a fast up-spike, which is related to the depolarization of the cell membrane, and a fast down-spike, which is related to the Na^+ currents through the small cleft between the membrane and the sensor surface. The elevation of exFP amplitude at the beginning of ischemia may be the result of stress-induced Na^+ influx.[32] The exFP amplitudes were progressively attenuated, indicating that the rate of depolarization and the extent of Na^+ influx signal were reduced as a direct result of ischemic insult (Figs. 2B and 3). A similar reduction in exFP amplitude has been demonstrated in other studies, suggesting K_{ATP} channel activation may be involved.[31,33]

A study has demonstrated that modification of cardiac electrical properties and propagation of action potential could lead to electrical instability and arrhythmias in acutely ischemic myocardium.[34] The propagation velocity, which is a reflection of conductivity and contractility of the cultured cardiomyocytes, was first elevated and then reduced after ischemic treatment (Fig. 2C). The present results are in line with previous studies, which have demonstrated the conduction velocity can be elevated as a result of an increase in extracellular potassium concentration (from 4 mM to 15 mM) during early ischemia.[35,36] Under ischemia or hypoxia, the spontaneous contractile activity of cardiomyocytes is progressively diminished because of depleting adenosyl triphosphate levels within cells. To maintain the membrane potential, adequate adenosyl triphosphate levels are required for both the formation of myosin–actin complex and the activation of Na^+-K^+ ATPase.[37] Another possible explanation for the reduced propagation velocity in ischemia is the alteration of gap junction protein expression. Intercellular communication through connexins allows the myocardium to work like a functional syncytium.[34] Connexin 43 is the most abundant connexin present in the myocardial tissue and its expression levels are associated with ischemic conditions.[16] There are two isoforms of Cx43 recognized by the total Cx43 antibody: a faster nonphosphorylated isoform (NP-Cx43) and a slower phosphorylated isoform (P-Cx43). Because the binding affinities of the antibody to different isoforms of Cx43 are unknown, the total Cx43 protein was measured instead. In this study, a slight elevation of the total Cx43 protein expression was observed 5 minutes after the initiation of ischemia with a more gradual reduction being observed thereafter (Fig. 6B). Although elevation of the total Cx43 protein expression in acute ischemia (within the first 15 minutes) has been demonstrated, no

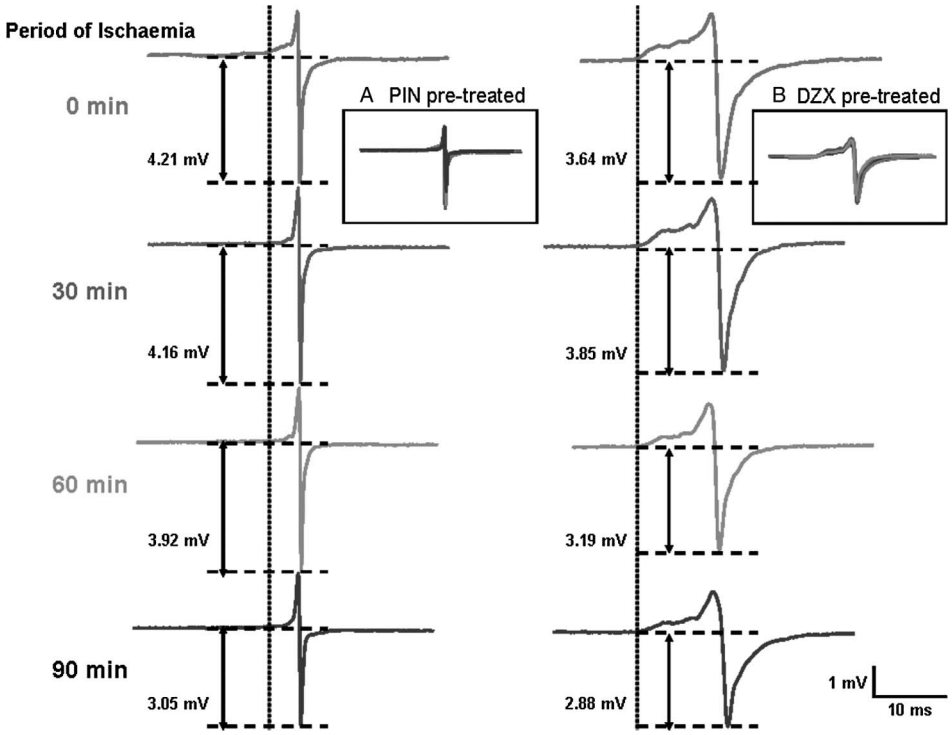

FIGURE 5. Representative traces of individual extracellular field potentials (exFP) of pinacidil (A, PIN) or diazoxide (B, DZX) pretreated rat cardiomyocytes under ischemia at different time points (0, 30, 60, and 90 minutes). The rate of depolarization and the duration of Na^+ influx signal were unchanged over the entire ischemic period (insets, A and B), but the extent of the Na^+ influx signal was reduced in both potassium channel openers (KCOs) pretreated cardiomyocytes at 90 minutes. Superimposition of traces obtained from all the time points are shown (insets, A and B).

conclusive explanations have yet been given.[19] However, in general, the total Cx43 reduces after 90 minutes of ischemia.[19] The reduction in Cx43 protein expression affects the electrical communication between cells, which probably explains the slower propagation velocity as observed in this study.

Cardiac research into the effects of KCOs preconditioning on cardiomyocytes has been carried out for decades, but long-term and real-time electrophysiology monitoring have not been carried out. In this study, the ischemia-induced electrophysiological changes were reduced when the cardiomyocytes were pretreated with KCOs before ischemia (Fig. 4A–C). There are many well-established models for studying the ischemic preconditioning effect on isolated cardiomyocytes; however, most of them tend to focus on the effects of cell viability and cell morphology.[27] By using the MEA, cardiac electrophysiology can be studied and its results correlated with the action potential measurements from isolated hearts.[38] The reductions in exFP amplitude suggested that the preconditioning effects of KCOs were the result of actions against the changes in membrane potential during ischemia. KCOs hyperpolarize the cardiomyocyte cell membrane and thus protect against depolarization induced by oxidative stress by reducing energy demand.[39] A reduction in Na^+ influx resulting from the elevation of extracellular K^+ has also been suggested.[33] The combined effects of KCOs on membrane potential and Na^+ influx suggest KCOs could reduce the overexcitation effect of cardiomyocytes induced by ischemia. A reduction in the extent of Na^+ influx in KCOs pretreated cells during ischemia was also demonstrated in the present study (Fig. 5).

The cardioprotection mechanism by the opening of K_{ATP} channels is not well understood. Thus far, three mechanisms have been proposed, including 1) inhibition of mitochondrial calcium uptake; 2) regulation of mitochondrial volume; and 3) modulation of reactive oxygen species.[40] Heinzel et al showed that a DZX-induced cardioprotective effect was not observed in Cx43-deficient mice, and the authors suggested that Cx43 may be part of the preconditioning signal cascade triggered at the level of mitoK_{ATP}.[41] In the present study, total Cx43 protein expression found in KCOs pretreated cardiomyocytes was lower, albeit insignificantly, compared with untreated cells (Fig. 6B). This may partly explain the reduction in propagation velocity in the KCOs pretreated cells when compared with the untreated cells under ischemia (Fig. 4Cc).

The sarcK_{ATP} channel is responsible for the shortening of action potential duration during acute ischemia, whereas the mitoK_{ATP} channel is primarily related to chronic ischemia.[13] The protective effect of PIN, and hence the significance of the sarcK_{ATP} channel, has been demonstrated in the present acute ischemic model. Because the present system can easily be adopted to accommodate a chronic ischemia model, our current plan is to investigate the long-term effect of ischemia and the cardioprotective effect of mitoK_{ATP} channel activation using the MEA. The MEA has the advantage of being able to perform long-term recordings of many cells as one syncytium over a long period of time, which is simply impossible in patch-clamp experiments.

CONCLUSION

The present study shows that distinctive electrophysiological differences can be observed between KCOs pretreated and untreated cells; and the extracellular electrophysiological changes can be correlated with cell viability and, to a small

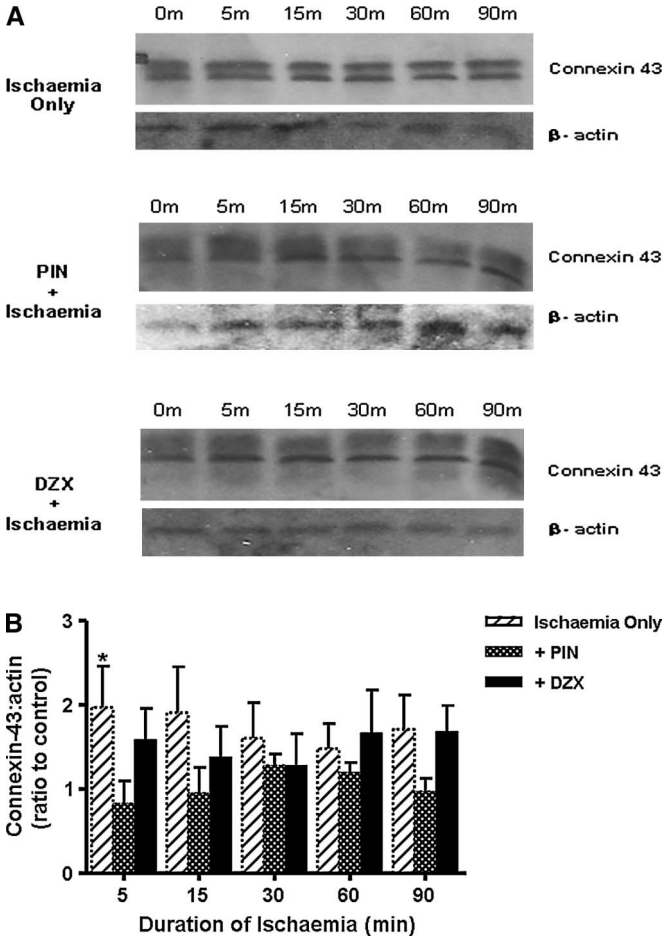

FIGURE 6. Effect of potassium channel openers (KCOs) on connexin 43 (Cx43) expression in rat cardiomyocytes under ischemic conditions. (A) Cultured cardiomyocytes were pretreated with 100 μM pinacidil (PIN) or diazoxide (DZX) three times for 5 minutes at 5-minute intervals before being subjected to 0, 5, 15, 30, 60, or 90 minutes of ischemia. At the end of each specified time point, samples were harvested, lysed, and immunoblotted with anti-Cx43 antibody. (B) Results of immunoblotting were determined by densitometry. Cx43 expression was elevated 5 minutes after ischemia (*$P < 0.05$), and then it reduced slightly over the course of ischemia. The Cx43 expression in KCOs pretreated cardiomyocytes was lower but remained constant under the same ischemic conditions. Results are means of three separate experiments.

extent, gap junction protein Cx43 expressions. Data collected from the present MEA model are in line with results from other common end-point study models. The protective effect by PIN is more prominent than DZX based on the present acute ischemia model. This further reiterates the significance of sarcK_{ATP} channel in acute ischemia.

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