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Differential Changes of ATP-sensitive Potassium Channel Current after Hypoxia-reperfusion Treatment in Mouse Neuroblastoma 2a (N2a) Cell

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Ischemic damage is one of the most serious problems. The openers of K_{ATP} channel have been suggested to have an effect to limit the ischemic damage. However, it is not yet clear how K_{ATP} channels of a cell correspond to hypoxic damage. To address the question, N2a cells were exposed to two different hypoxic conditions as follows: 6 hours hypoxia followed by 3 hours reperfusion and 12 hours hypoxia followed by 3 hours reperfusion. As the results, 6 hours hypoxic treatment increased glibenclamide-sensitive basal K_{ATP} current activity (approximately 6.5-fold at 0 mV test potential) when compared with nomoxic condition. In contrast, 12 hours hypoxic treatment induced a relatively smaller change in the K_{ATP} current density (2.5-fold at 0 mV test potential). Additionally, in experiments where K_{ATP} channels were opened using diazoxide, the hypoxia for 6 hours significantly increased the current density in comparison to control condition ($p<0.001$). Interestingly, the augmentation in the K_{ATP} current density reduced after exposure to the 12 hours hypoxic condition ($p<0.001$). Taken together, these results suggest that K_{ATP} channels appear to be recruited more in cells exposed to the 6 hours hypoxic condition and they may play a protective role against hypoxia-reperfusion damage within the time range.

Key Words: K_{ATP} channel, Hypoxia, Diazoxide, Glibenclamide

INTRODUCTION

Ischemic injury in brain is one of the most serious medical problems. Substantial evidence has suggested that glutamate is an important mediator of ischemic brain injury. (Choi, 1988; Krieglstein et al, 1994). Over the past years, numerous selective glutamate receptor blockers have been developed and tested for their ability to limit the deleterious consequences of cerebral ischemia. (Krieglstein et al, 1994). Additionally, therapeutic strategies have been developed to inhibit pathophysiological reactions that occur downstream of glutamate receptor activation. (Bartus et al, 1994; Krieglstein et al, 1994). Alternative approaches have been suggested that ATP-sensitive potassium channels (K_{ATP}) might play an important role in hypoxia and ischemia-induced glutamate release (Noma, 1983). In general, potassium channels are known to play important roles in regulation of membrane potential. The regulation of potassium channel activity is implicated in the overall neuronal response and adaptation to O_2 deprivation (Belousov et al, 1995).

Neurons express more than four different subfamily of K^+ channel including voltage-sensitive K^+ channel, Ca^{2+}-activated K^+ channel, inward rectifier (K_{IR}) K^+ channels and K_{ATP} channels. The pore-forming proteins of both K_{IR} and K_{ATP} channels belong to the same structural family, the inwardly rectifying channel family (K_{IR}) although they have different subfamilies (Liss et al, 2001). Since inward rectification was first described in skeletal muscle by Katz in 1949, inward rectifier K^+ channels have been identified in many other tissues where they have important functions in maintaining the resting potential. K_{ATP} channels were discovered recently in a variety of cells such as cardiac muscle cells pancreatic cells, skeletal muscle, and neurons. They have the general function of linking membrane K^+ permeability of cells to their metabolic state and are thus sensitive to intracellular ATP (Quayle et al, 1997). These channels are inhibited by high concentrations of intracellular ATP, insensitive to changes in intracellular Ca^{2+}, activated by certain K^+ channel agonists such as pinacidil, cromakalim, and diazoxide (DZX). Glibenclamide (GBC) is a known selective K_{ATP} channel blocker at submicromolar concentrations (Bray & Quast, 1992; Edwards & Weston, 1993; Teramoto & Brading, 1996). Several studies have shown that in brain models of ischemia-reperfusion, K_{ATP} channel openers such as cromakalim and diazoxide have protective effects (Heurteaux, 1993; Reshef, 1998) that could be eliminated by glibenclamide (Grover et al, 1995). These channels are activated when the intracellular level of ATP drops, as it occurs during hypoxia and ischemia. Activation of these channels hyperpolarizes neurons, which leads to inhibition of neuronal excitability and reduction of noxious

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ABBREVIATIONS: GBC, glibenclamide; DZX, diazoxide; ATP, adenosine triphosphate; K_{ATP} channel, ATP-sensitive potassium channel; KIR channel, inward rectifier potassium channel.

neurotransmitters release. Activation of K^+ channels is therefore assumed to be an endogenous defense mechanism against cerebral hypoxia and ischemia by prolonging the period before membrane depolarization occurs (Lindeman et al, 1994). However, it is not yet clear whether the K_{ATP} channel currents are regulated during the progress of hypoxic condition. In this study, it was shown that current density of K_{ATP} channel was changed dependent on duration of exposure to a hypoxic condition in mouse neuroblastoma cells.

METHODS

Cell culture

Mouse neuroblastoma 2a (N2a) cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air-5% CO_2 incubator at 37°C. The cells at 80% confluence in 35 mm culture dishes, were harvested by trypsinization and replaced onto three culture dishes. When the cell population reached to 80% confluence, the plates were used for hypoxic-reperfusion treatment.

Hypoxia-reperfusion treatment

Hypoxia was induced using a hypoxic chamber (Forma, USA) containing 85% N_2, 5% H_2 and 10% CO_2. Dishes were returned to a normal incubator and kept for 3 hours to mimic reperfusion. In this experiment, the cells were exposed to hypoxia for two different durations (6 hours and 12 hours) with a fixed reperfusion time of 3 hours. The patch-clamp recordings were performed within two hours after the treatment and there were no significant differences between early and late recordings.

Patch-clamp experiment

Before recordings, the culture medium was washed with normal saline thoroughly and then replaced with a high barium containing solution. All solution was aerated with 95% O_2 and 5% CO_2 gas during experiment. The K_{ATP} currents were recorded using the whole-cell variant of the patch clamp technique. All chemicals used in experiments were obtained from Sigma Chemical Co. (St Louis, MO) except the items mentioned. The normal saline contained 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and 5 mM Glucose. High barium solution contained 110 mM $BaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.4). The internal solution contained 10 mM NaCl, 102 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.1 mM Na_2-ATP, 1 mM Na_2-GTP and 10 mM EGTA (pH 7.2). All solutions were filtered through 0.22 μm syringe filters. Drugs were applied externally by gravity-controlled bath perfusion (1 ml/min). The cell membrane capacitance and series resistance were compensated (>80%) electronically using an EPC-9 amplifier (HEKA Electronik, Lambrecht, Germany). The K_{ATP} channel currents were sampled at 0.5 kHz and low-pass filtered at 2 kHz (−3dB). To eliminate errors from cell size differences, current amplitude was converted to current density (pA/pF) by dividing a membrane capacitance. Data analysis was performed using the Pulse/Pulsefit (v8.50, Heka), Excell 2000 (Microsoft, v9.0) and Sigma Plot 2000 (SPSS Inc., v6.0) software. Data were presented as mean value±SEM. Statistical significance was evaluated using one-way analysis of variance (ANOVA). $P<0.05$ was considered significant. All experiment was carried out at room temperature (20~24°C).

RESULTS

Detection of ATP sensitive-potassium channel currents in mouse neuroblastoma 2a cells

To reduce contamination from other potassium channel currents, the external bathing solution was maintained with a 110 mM high Ba^{2+} contained saline. Membrane currents were evoked by test pulses for 200 ms between −80 mV to +10 mV in a 10 mV increment from a holding potential of −60 mV. To confirm the presence of K_{ATP} currents in mouse neuroblastoma 2a cells, a K_{ATP} channel opener, diazoxide or a blocker, glibenclamide was applied. As illustrated in Fig. 1, bath application of diazoxide (10 μM) enhanced the step currents in an untreated control cell. Conversely, glibenclamide (10 μM) prevented the effect of diazoxide and further reduced the basal currents, suggesting that K_{ATP} channels were basally active under the recording conditions (Fig. 1).

Dependence of K_{ATP} channel activity on duration of exposure to hypoxia

To assess the effects of hypoxia on the K_{ATP} channel activity, K_{ATP} currents were isolated by subtracting glibenclamide-insensitive currents and normalized with the cell capacitance. Fig. 2 shows summary of the changes in the K_{ATP} current (A, glibenclamide-sensitive basal current; B, diazoxide-sensitive current+glibenclamide-sensitive basal current) density in cells exposed to two different hypoxic conditions. The hypoxia for 6 hours significantly increased

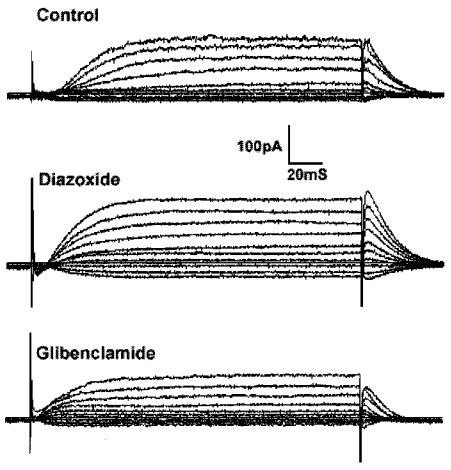

Fig. 1. Current changes in nomoxia by selective K_{ATP} channel effectors. Upper trace: Control condition. Middle trace: Diazoxide (DZX, 10 μM) treatment. Bottom trace: Glibenclamide (GBC, 10 μM) treatment. Treatment of a selective K_{ATP} channel opener, diazoxide, reveals typical potassium current. A selective K_{ATP} channel blocker, glibenclamide, treatment takes away the effect of diazoxide treatment by further reducing the currents.

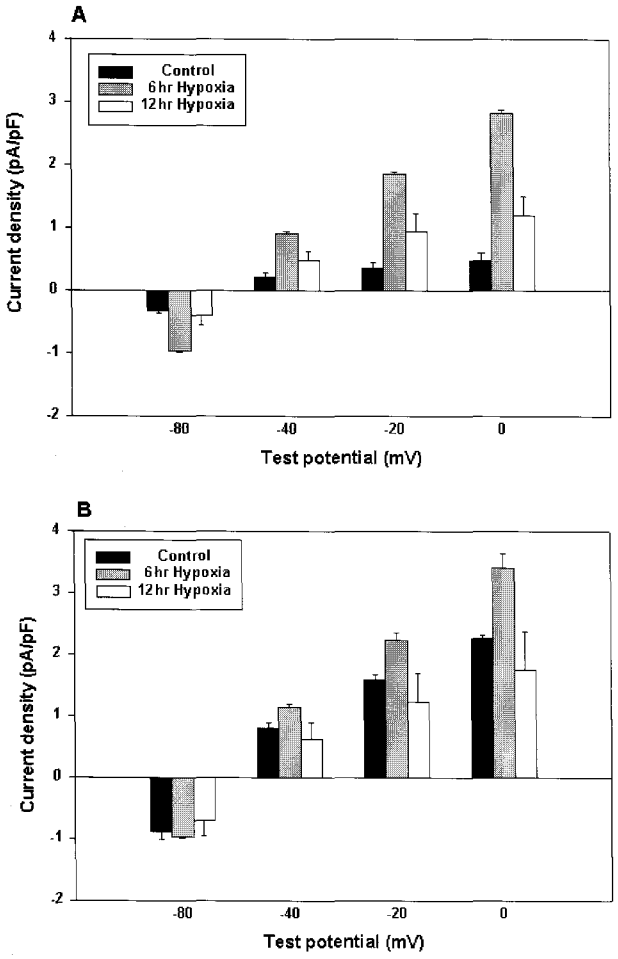

Fig. 2. Changes in K_{ATP} channel current density. Subtracting glibenclamide insensitive currents from either untreated or diazoxide treatment cases resulted the current density. A: Glibenclamide-sensitive basal current density. At 0 mV test potential, it is clear that 6hr hypoxia condition (2.82±0.05 pA/pF, n=6, 592.97%, $p<0.05$) has relatively larger increase of current density than the 12 hr hypoxia condition (1.20±0.29 pA/pF, n=7, 252.39%, $p<0.05$) case. Nomoxia condition has relatively low current density (0.48± 0.13 pA/pF at 0 mV test potential, n=6). B: Diazoxide plus glibenclamide sensitive basal current density. At 0 mV test potential, the current density change is noticeably increased in the 6 hr hypoxia condition (3.42±0.22 pA/pF, n=6, 151.17%, $p<0.001$), but it shows less change in the 12 hr hypoxia condition (1.75±0.62 pA/pF, n=7, 77.33%, $p<0.001$) than in control case (2.26±0.07 pA/pF, n=7).

K_{ATP} current density measured at different test potentials when compared with nomoxia (Fig. 1A and B, $p<0.05$ and $p<0.001$ respectively). Conversely, the K_{ATP} current density after exposure to the hypoxic condition for 12 hours was even smaller than the control value (Fig. 2B, 77.33%, $p<0.001$).

DISCUSSION

K_{ATP} channels found in many types of excitable cells are believed to provide a link between excitability and metabolic status in peripheral and central nervous system (Ashcroft & Rorsman, 1990). Activation of K_{ATP} channels induces hyperpolarization, decreases membrane excitability, and reduces O_2 consumption (Belousov et al, 1995). A recent study has shown that K_{ATP} currents are involved in neuronal response to hypoxia or ischemia (Chi & Xu, 2000).

In this study, it was addressed whether the K_{ATP} channel currents are regulated during the progress of hypoxic condition. In this regard, K_{ATP} channel currents were isolated by the treatment of diazoxide or glibenclamide, a selective K_{ATP} channel opener and blocker, respectively in a whole cell-ruptured configuration. In comparison to the control condition (nomoxia), the 6 hours hypoxic treatment significantly increased the K_{ATP} channel current density. When the cells were exposed to the 12 hours hypoxic condition, however, the current density was declined to the control level. These data suggest that the hypoxia-induced changes in K_{ATP} channel currents are time-specific and consequently the protection effects by K_{ATP} channel activity might be limited to the early stage of hypoxia. At this time, it remains unclear how the K_{ATP} current density were increased during the progress of hypoxia. One plausible explanation is that expression of K_{ATP} channels is up-regulated during hypoxia in a time-dependent manner. In conclusion, hypoxia induces time-specific changes of K_{ATP} channel currents in mouse neuroblastoma 2a cells. However, in the next study, it needs to be investigated molecularly the time-dependent regulation of K_{ATP} channels during hypoxia. Furthermore, variant time scaled experiments needs to be designed for obtaining more conclusive results.

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