Short Communication

A Novel Opener of Large-Conductance Ca^{2+} -Activated K^+ (BK) Channel Reduces Ischemic Injury in Rat Cardiac Myocytes by Activating Mitochondrial K_{Ca} ChannelKazuho Sakamoto^{1,2}, Susumu Ohya¹, Katsuhiko Muraki³, and Yuji Imaizumi^{1,*}¹Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, Nagoya 467-8603, Japan²Department of Pharmacology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan³Laboratory of Cellular Pharmacology, School of Pharmacy, Aichi Gakuin University, Nagoya 464-8650, Japan

Received June 13, 2008; Accepted July 7, 2008

Abstract. It has been suggested that a new type of large-conductance Ca^{2+} -activated K^+ (BK) channel is distributed in the inner mitochondrial membrane (mito K_{Ca} channel) and that its opening may attenuate ischemic cardiac injury. We examined effects of 12,14-dichlorodehydroabietic acid (diCl-DHAA), a novel BK-channel opener, on rat cardiac myocytes and mitochondria. Application of diCl-DHAA concentration-dependently reduced Ca^{2+} overload in isolated mitochondria, activated mito K_{Ca} channels in inside-out patches of mitochondrial membrane, facilitated flavoprotein-oxidization in myocytes, and increased cellular viability under simulated ischemia. In conclusion, diCl-DHAA directly opens mito K_{Ca} channels, prevents Ca^{2+} influx into matrix, and reduces ischemic injury in cardiac myocytes.

Keywords: mitochondria, large-conductance Ca^{2+} -activated K^+ (BK) channel, cardiac ischemia

In analogy to mitochondrial ATP-sensitive K^+ (mito K_{ATP}) channels (1), mitochondrial Ca^{2+} -activated K^+ (mito K_{Ca}) channels are considered to be expressed in the inner mitochondrial membrane (IMM) (2). The K^+ entry through mitoK channels depolarized mitochondrial membrane potential ($\Delta\psi_{\text{m}}$), which reduces the driving force for Ca^{2+} influx into mitochondria. Thus the activation of mitochondrial K^+ channels inhibits Ca^{2+} overload in mitochondria (3). Correspondingly, an opener of the mito K_{Ca} channel, NS-1619, is effective to protect cells against ischemic injury in a similar manner as mito K_{ATP} -channel openers (e.g., diazoxide and pinacidil) (2, 3). Previously, we have reported that 17 β -estradiol activates mito K_{Ca} channels and may protect cardiac myocytes from ischemic damage (4).

This study was undertaken to clarify whether the novel large-conductance Ca^{2+} -activated K^+ (BK)-channel opener 12,14-dichlorodehydroabietic acid (diCl-DHAA)

(Fig. 1A) (5), which has a molecular structure apparently different from those of NS-1619 and 17 β -estradiol, shows mito K_{Ca} -opening action and cardioprotective effects. The present results give additional evidence that mito K_{Ca} openers, including diCl-DHAA, are potential candidates for a new class of cardiac protective drugs targeted to mito K_{Ca} channels.

For the isolation of ventricular myocytes from rat hearts, the purification of mitochondrial fractions from rat cardiac tissue and the formation of mitoplasts were prepared as described (4).

Mitochondrial Ca^{2+} handling was measured at 25°C as the change in the free Ca^{2+} concentration (extra-mitochondrial Ca^{2+} concentration: $[\text{Ca}^{2+}]_{\text{em}}$) of the following solution (in a cuvette): 110 mM KCl, 5 mM K_2HPO_4 , 5 mM pyruvate, 5 mM malate, and 10 mM MOPS (adjusted to pH 7.2 with KOH). We used Calcium Green-5N (1 μM) as a $[\text{Ca}^{2+}]_{\text{em}}$ indicator, which was excited at 506 nm. Emitted fluorescence was monitored at 532 nm with an F-4500 spectrofluorometer (Hitachi, Tokyo). The fluorescence intensity was converted into $[\text{Ca}^{2+}]_{\text{em}}$ by the following equation:

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Published online in J-STAGE on August 30, 2008 (in advance)
doi: 10.1254/jphs.081505C

$[Ca^{2+}]_{em} = K_d(F - F_{min}) / (F_{max} - F)$, where F is the fluorescence intensity of Calcium Green-5N in the experiment, F_{min} is the fluorescence in the presence of 1 mM EGTA, F_{max} is the fluorescence in the presence of 10 mM Ca^{2+} , and K_d is dissociation constant of Calcium Green-5N (14 μ M).

The autofluorescence of flavoprotein in cardiac myocytes was measured as an indirect indicator of mitochondrial K^+ channel activity and the fluorescence of tetramethylrhodamine ester (TMRE), as a $\Delta\psi$ m indicator (4). To determine effects of diCl-DHAA on cell injury following hypoxia, we adopted the Simulated Ischemia method and measured cell viability with trypan blue as previously described (4).

For single mitoK_{Ca} channel current recording, the inside-out patch clamp was applied to mitoplasts using an EPC-7 amplifier (List, Darmstadt, Germany) and analyzed as reported previously (4). The pipette solution contained 29.5 mM KCl, 110.5 mM K-glutamate, 10 mM HEPES, 1 mM EGTA, and 0.76 mM $CaCl_2$. The bathing solution contained 140 mM KCl, 1 mM EGTA, and 0.76 mM $CaCl_2$. In both solutions, pCa and pH were adjusted to 6.3 and 7.2, respectively.

Data values are each expressed as the mean $\pm$ S.E.M. in the text. Statistical significance between two or among multiple groups was examined using Student's t or Tukey's test after the F -test or one-way ANOVA, respectively. Significance was expressed in the figures by * P <0.05 or ** P <0.01.

We first investigated whether diCl-DHAA modulates the capability of mitochondrial Ca^{2+} storage, a key factor of cell death. As shown in Fig. 1B, the cardiac mitochondria fraction (1.5 mg-protein/ml) absorbed injected 30 μ M Ca^{2+} . The following application of 3 μ M diCl-DHAA drained Ca^{2+} from mitochondria. DiCl-DHAA-induced Ca^{2+} release occurred in a concentration-dependent manner (Fig. 1C). We estimated Ca^{2+} release as the rate of increase in $[Ca^{2+}]_{em}$. In the absence and presence of 3 μ M diCl-DHAA, the rate of increase in $[Ca^{2+}]_{em}$ was 0.042 ± 0.016 and 0.452 ± 0.058 μ M/s (P <0.01), respectively. NS-1619 (3 μ M) also drained Ca^{2+} from the Ca^{2+} -loaded mitochondria (0.164 ± 0.028 μ M/s, P <0.05) (Fig. 1D). The results indicate that diCl-DHAA drains the Ca^{2+} -storage function and prevents Ca^{2+} overload of isolated cardiac mitochondria.

Mitochondrial Ca^{2+} uptake depends on $\Delta\psi$ m. Thus, we examined whether diCl-DHAA modifies $\Delta\psi$ m. Cardiomyocytes, loaded with 3 μ M TMRE, displayed non-smear staining. In these conditions, fluorescent intensity of TMRE was inversely related to $\Delta\psi$ m. Application of 3 μ M diCl-DHAA also increased the fluorescence intensity and addition of 3 μ M paxilline, a selective BK- and mitoK_{Ca}-channel blocker, reduced the

Fig. 1. DiCl-DHAA drained Ca^{2+} from overloaded cardiac mitochondria. **A:** Chemical structure of diCl-DHAA. **B:** Representative changes in extra mitochondrial Ca^{2+} concentration measured by Calcium Green-5N. **C:** Ca^{2+} release induced by 0.3, 1, and 3 μ M diCl-DHAA from mitochondria preloaded with 30 μ M Ca^{2+} . The arrow indicates application of diCl-DHAA. **D:** Summarized data from 4 trials for the effects of diCl-DHAA and NS-1619 (NS) on the rate of mitochondrial Ca^{2+} release. * P <0.05, ** P <0.01 vs Control.

intensity. The relative fluorescent intensity of TMRE (F/F_0) in the presence of diCl-DHAA and following the addition of paxilline was 1.035 ± 0.008 and 0.943 ± 0.015 (P <0.01, $n = 6$), respectively (data not shown).

Fig. 2. Effect of diCl-DHAA on flavoprotein autofluorescence. A: Transmitted image (a) and autofluorescence images with pseudo-colors from a single ventricular myocyte were obtained using the HiSCA/Argus imaging system (b–f). The autofluorescence intensity was low in blue and high in red as indicated in the bar. B: The time course of changes in fluorescence intensity (%) in a cell exposed to 3 μ M diCl-DHAA and/or 3 μ M PAX. At the end of experiment, the flavoprotein autofluorescence was normalized for 0 and 100% flavoprotein fluorescence: respiration was blocked by 3 mM NaCN to fully reduce the fluorescence and then uncoupled by 100 μ M DNP to achieve the maximal fluorescence. The letters “b–f” shown in the time course correspond to those of images in panel A. C: Summarized data obtained in experiments typically shown in panel B. Addition of diCl-DHAA significantly increased flavoprotein oxidation (** P <0.01 vs Control, n = 6). The addition of PAX reversed flavoprotein oxidation to a level close to the control (** P <0.01 vs diCl-DHAA, n = 6).

These results revealed that diCl-DHAA depolarized $\Delta\Psi_m$ in rat cardiac myocytes.

Depolarization of $\Delta\Psi_m$ induces compensatory activation (oxidation) of electron-transfer flavoprotein to increase its autofluorescence. Thus, effects of diCl-DHAA on mitoK_{Ca} channels were indirectly evaluated by measuring flavoprotein-autofluorescence. Figure 2A shows typical images of flavoprotein-autofluorescence in a myocyte exposed to 3 μ M diCl-DHAA and/or 3 μ M paxilline. After the incubation under control conditions, 3 μ M diCl-DHAA and 3 μ M paxilline were added sequentially. At the end of each experiment, the flavoprotein-autofluorescence was normalized for minimal and maximal flavoprotein fluorescence (Fig. 2B). As summarized in Fig. 2C, diCl-DHAA increased flavoprotein oxidation from $5.6 \pm 1.0\%$ to $13.7 \pm 0.8\%$ (P <0.01 vs Control, n = 6). Addition of 3 μ M paxilline reversed flavoprotein oxidation to $7.1 \pm 0.8\%$ (P <0.01 vs Control, n = 6). The results indirectly indicate that diCl-DHAA activates mitoK_{Ca} channels.

Effects of diCl-DHAA on single mitoK_{Ca} currents were examined in the inside-out patch clamp mode. In 20% of excised membrane patches, we observed a large conductance channel with 260-pS (259.3 ± 16.6 pS, n = 4) (Fig. 3: A and B). Currents having some different

conductances often appeared and they may be due to sub-conductance states of mitoK_{Ca} currents (2) or contamination of other ion channels in the membrane (4). Since 260-pS current was blocked by 3 μ M paxilline, we identified the 260-pS channel as the mitoK_{Ca} channel. The application of 3 μ M diCl-DHAA increased the open probability (NP_o) of 260-pS current from 0.00053 ± 0.00019 to 0.02377 ± 0.00361 (P <0.01, at +40 mV). The Ca²⁺ concentration of bathing solution, to which the patch membrane was exposed ([Ca²⁺]_m), was adjusted to 500 nM (Fig. 3: C and D). Addition of 3 μ M paxilline eliminated the channel opening events. These results confirm that diCl-DHAA activates mitoK_{Ca} channel as well as BK channel.

We examined that protective effects of diCl-DHAA on cardiomyocytes against ischemic cell death by using the simulated ischemia method. After 30 min of ischemia, the lethality of cardiac myocytes in the control group was $67.3 \pm 3.2\%$. In the presence of 3 μ M diCl-DHAA, the lethality was decreased to $29.3 \pm 5.9\%$ (P <0.01), whereas addition of 3 μ M paxilline removed the cytoprotection by diCl-DHAA ($56.3 \pm 3.2\%$, P <0.01 vs diCl-DHAA). On the other hand, after 120 min of ischemia, the protective effect was not significant ($83.8 \pm 3.5\%$ in the control group and $68.5 \pm 7.7\%$ in the

Fig. 3. Identification of single mitoK_{Ca} channel currents in rat heart and effects of diCl-DHAA on mitoK_{Ca} channels. **A:** Original single channel current traces of cardiac mitoK_{Ca} channels. Recordings were obtained from the mitoplast patch at -40 , -20 , 0 , $+20$, and $+40$ mV in the inside-out patch configuration in symmetrical 140 mM K⁺ conditions. The arrowheads indicate the zero current level. **B:** The relation between unitary current amplitude and test potentials was plotted in a range of -60 and $+60$ mV. The single channel conductance was determined from the slope ($n=4$). **C:** Effects of 3 μ M diCl-DHAA and 3 μ M paxilline (PAX) were recorded at $+40$ mV. **D:** Summarized data demonstrating open probability (NP_o) of mitoK_{Ca} channels. NP_o was calculated from the histogram as the relative time spent in the open state. Applications of diCl-DHAA significantly increased NP_o (** $P<0.01$ vs Control, $n=3$). The addition of PAX abolished channel activities (** $P<0.01$ vs diCl-DHAA, $n=3$).

diCl-DHAA group, respectively, $P>0.05$). The results indicate that diCl-DHAA attenuates ischemic damage presumably by opening mitoK_{Ca} channels and thereby delayed the cell death.

The present study clearly indicates that diCl-DHAA shows protective effects on cardiac myocytes against ischemic injury via the opening of mitoK_{Ca} channels and gives distinct evidence supporting that mitoK_{Ca}-channel opening is a novel way to protect cardiac myocytes from ischemic and re-perfusion injury.

BK channels are expressed in the plasma membrane of various types of excitable tissues but not in the plasma membrane of mammalian cardiac myocytes (6). In these excitable cells, BK channels act as one of the obligatory factors in negative-feedback regulation of intracellular Ca²⁺ concentration because BK channel opening attenuates Ca²⁺ influx through voltage-dependent Ca²⁺ channels by membrane hyperpolarization (7). In contrast, mitoK_{Ca} channels may be functionally expressed in IMM and may take part in ischemic preconditioning in analogy to mitoK_{ATP} channels (3). A line

of evidence suggesting that mitoK_{Ca} opening may contribute to protection of myocardium from ischemic injury has been progressively accumulated (2, 8, 9). Although several reports have shown that NS-1619 possesses a protective effect against infarction, presumably by opening mitoK_{Ca} channels, direct evidence supporting the opening of mitoK_{Ca} channels by NS-1619 is quite limited. Some reports imply that NS-1619 might activate non-selective cation influx in IMM (10, 11). In this study, we clearly demonstrated that diCl-DHAA opens mitoK_{Ca} channels by using the inside-out patch clamp configuration.

There are still arguments about the regulation of $\Delta\Psi$ m by mitoK_{ATP} channel; its opening by diazoxide decreased $\Delta\Psi$ m in some studies (12), but not others (13). In a study on mitoK_{Ca}-channel opener, NS-1619 invoked a decrease in $\Delta\Psi$ m (11) and oxidation of flavoprotein (14). Previously, we also reported that application of NDGA, a BK-channel opener, also induced depolarization of $\Delta\Psi$ m in porcine coronary artery smooth muscle cells (15). In this study, results about the effects of diCl-

DHAA were consistent with those of NS-1619. Therefore, diCl-DHAA and NS-1619 share these effects presumably due to mitoK_{Ca}-channel opening, while diCl-DHAA is more potent than NS-1619.

In conclusion, our data indicate that i) diCl-DHAA, a potent BK-channel opener, activates mitoK_{Ca} channels directly, ii) diCl-DHAA-induced opening of mitoK_{Ca} channels depolarizes $\Delta\psi_m$, iii) this depolarization prevents Ca²⁺ influx into matrix, iv) diCl-DHAA is effective in protection of cardiac myocytes from ischemic injury. Opening of sarcolemmal K⁺ channels under ischemic condition is occasionally harmful because of the risk of lethal arrhythmias. In the case of a mitoK_{Ca}-channel opener, there is no such a risk because BK channels are not functionally expressed in sarcolemma of cardiac myocytes (7). Therefore, drugs targeting the mitoK_{Ca} channel are thought to be useful and attractive as a new therapeutic tool for cardiac protection from injury following ischemia and reperfusion. DiCl-DHAA is a useful tool to study the role of mitoK_{Ca} opening in cardiac protection.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (20056027) from The Ministry of Education, Culture, Sports, Science, and Technology and by a Grant-in-Aid for Scientific Research (B) (20390027) from Japan Society for the Promotion of Science to YI.

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