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Increased Expression of ATP-sensitive K^+ Channels Improves the Right Ventricular Tolerance to Hypoxia in Rabbit Hearts

Seong Woo Choi[1,*], Jun Seok Ahn[1,*], Hyoung Kyu Kim[1], Nari Kim[1], Tae-Hoon Choi[2], Sung-Woo Park[3], En A Ko[1], Won Sun Park[4], Dae-Kyu Song[5,#], and Jin Han[1,#]

[1]National Research Laboratory for Mitochondrial Signaling, Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, Inje University, Busan 614-735, [2]Department of Sport and Leisure Studies, Andong Science College, Andong 760-709, [3]Department of Neuropsychiatry, College of Medicine, Paik Inje Memorial Clinical Research Institute, Inje University, Busan 614-735, [4]Department of Physiology, School of Medicine, Kangwon National University, Chuncheon 200-701, [5]Department of Physiology & Chronic Disease Research Center, Keimyung University School of Medicine, Daegu 700-712, Korea

ATP-sensitive K^+ channels (K_{ATP}) are major component of preventing ischemia-reperfusion injury. However, there is little information regarding to the expressional difference of K_{ATP} and its function between left and right ventricles. In this study, we measured the lactate dehydrogenase release of rabbit heart slices in vitro and determined the difference of the K_{ATP} expression at the both ventricles by measuring the level of K_{ATP}-forming Kir6.2 (OcKir6.2) mRNA using in situ hybridization. The hearts were preconditioned with 15 min hypoxia and reoxygenated for 15 min before a hypoxic period of 60 min, followed by reoxygenation for 180 min. With hypoxic preconditioning (100% N_2) with 15 min, left ventricles (LV) showed higher release of LDH comparing with right ventricles (RV). Adding K_{ATP} blocker glibenclamide (10 μM) prior to a hypoxic period of 60 min, hypoxic preconditioning effect of RV was more abolished than LV. With in situ hybridization, the optical density of OcKir6.2 was higher in RV. Therefore, we suggest that different K_{ATP} expression between LV and RV is responsible for the different response to hypoxia and hypoxic preconditioning of rabbit hearts.

Key Words: ATP-sensitive K^+ channels, Kir6.2, Right ventricle, Hypoxia

INTRODUCTION

The anatomical differences between the right and left ventricle are well known. As the left ventricle is exposed to systemic circulatory workloads, the myocardium is thicker and denser than that in the right ventricle. The coronary artery, which provides oxygen and nutrients to the heart, is variable among animal species. The rabbit heart is a good model in which to study coronary hypoxia as the left coronary artery dominantly provides blood to the left ventricle, which is different from the human heart in which the right coronary artery partially supplies blood to the left ventricle [1]. Ischemic damage occurs if perfusion of the coronary artery is down-regulated. The heart induces ischemic preconditioning (IPC) to prevent reperfusion injury. Baker et al concluded that memory of the cardioprotective effects of normoxia in the immature rabbit heart is not congenital and that ATP-sensitive-potassium (K_{ATP}) channels are strongly associated with this acquired ischemic preconditioning trait [2-4].

K_{ATP} channels have been identified in a range of animal tissues, including the pancreas, blood vessels, brain, retina, and heart [5]. In myocardial preconditioning, the surface or sarcolemmal K_{ATP} channels are a major component involved in triggering or mediating IPC. In addition, it has recently been reported that mitochondrial K_{ATP} channels participate in IPC [5,6]. K_{ATP} channels consists of a cellular membrane with pore-forming rectifying Kir6.x subunits and sulfonylurea receptors. Two dominant Kir6.x subunits have been identified to date, *i.e.*, Kir6.1 and Kir6.2. In rabbit cardiomyocytes, the dominant subtype is Kir6.2. It has been suggested that the cardiac sarcolemmal K_{ATP} channels are composed of an octameric complex of two types of subunit, *i.e.*, Kir6.2 and SUR2A subunits [5]. Kir6.2 is also a crucial subunit in the K^+ Surface K_{ATP} channel [7]. Zingman reported that there were impaired adaptive cardiac responses to adrenergic stress (isoproterenol) in Kir6.2 knockout mice, and left ventricular diastolic pressure was critically decreased in comparison with wild-type controls [8]. There have been numerous studies of ventricular function *in vivo*. Although there have been previous electrophysiological evaluations of K_{ATP} channels, these studies only focused on the left ventricle. However, in this study

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Corresponding to: Jin Han, National Research Laboratory for Mitochondrial Signaling, Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, Inje University 633-165, Gaegeum-dong, Busanjin-gu, Busan 613-735, Korea. (Tel) 82-51-890-6727, (Fax) 82-51-894-5714, (E-mail) phyhanj@inje.ac.kr
*Both authors contributed equally to this work.
#Both authors contributed equally to this work.

ABBREVIATIONS: IPC, ischemic preconditioning; K_{ATP}, ATP-sensitive potassium; OcKir6.2, rabbit Kir6.2; NT, normal Tyrode's.

we examined the expressional difference of K_{ATP} channels between the left and right ventricles. We determined the nucleotide sequence and deduced the amino sequence of rabbit Kir6.2 (OcKir6.2). Northern hybridization and *in situ* hybridization analyses revealed differential Orkit6.2 mRNA expression between left and right ventricular cardiomyocytes of the New Zealand white rabbit (*Oryctolagus cuniculus*).

METHODS

Animals

Male New Zealand White Rabbits weighing less than 1 kg were used in this study. The rabbits were housed one per each cage in a room maintained at 21°C with a 12-h/12-h light/dark cycle. Food and water were available *ad libitum*. All animal handling was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and the Korean Academy of Medical Science. All experiments involving animals were approved by the Committee for Animal Experimentation and the Institutional Animal Experimentation and Institutional Animal Laboratory Review Board of Inje Medical College. Rabbits were sacrificed using a mixture of pentobarbital (50 mg/kg, i.v.) and heparin (100 U/kg, i.v.).

Single cell isolation

Rabbit heart was cannulated and then retrogradely perfused via Langendorff apparatus. After removal of blood in the heart, it was perfused with enzyme solution containing 0.01% of *Yakult* collagenase for 20～25 min. Following the isolation procedure, right and left ventricles were dissected and each ventricular myocyte was obtained by gentle agitation in storage solution contained (in mM): KOH 70, L-glutamate 50, KH_2PO_4 20, KCl 55, taurine 20, $MgCl_2$ 3, glucose 20, HEPES 10, EGTA 0.5, adjusted to pH 7.3 with KOH.

In situ hybridization

In situ *hybridization* was performed as follow previously established techniques [9]. Briefly, heart sections were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. Acetylation was performed in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) at room temperature for 10 min. Sense and antisense RNA probes corresponding to cDNA fragments of OcKir6.2 were inserted into the pGEM-T easy vector (Promega, Madison, WI) and labeled with digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, IN) using T7 or SP6 polymerase. Ethanol was used for dehydration and the sections were hybridized in a buffer consisting of 50% deionized formamide, 300 mM NaCl, 1×Denhardt's solution, 50 mM Tris-HCl (pH 6.0), 2 mM EDTA, 10% dextran sulfate, and 0.25 mg/ml yeast tRNA with 200 ng/ml of either antisense or sense BDNF cRNA probe. Hybridization was performed overnight at 58°C in a humidified chamber with 50% deionized formamide/4×SSC. After RNase A (20 μg/ml) treatment at room temperature, the sections were washed through serial post-hybridization steps, including a shaking water bath starting in 2×SSC and ending with high-stringency washing in 0.1×SSC at 60°C. The bound probe was detected using alkaline phosphatase-conjugated anti-digoxigenin antibody and developed in BCIP/ NBT solution using a DIG Detection kit.

Electrophysiological recording in isolated rabbit ventricular myocytes

Whole-cell currents were recorded at room temperature using whole-cell voltage-clamping patch clamp configuration. Data was collected and saved with an Axopatch 1D amplifier (Axon instruments, Union, CA) and Patchpro 1.0 software, developed by our lab. K_{ATP} currents were evoked by specific opener, pinacidil and were recorded from a holding potential of 70 mV using a ramp (0.5 dV/dt) pulse protocol from 120 to+60 mV, repeated every 10 second. The pipette solution contained (in mM): K-Aspartate 105, KCl 25, NaCl 5, $MgCl_2$ 3, Mg-ATP 0.1, BAPTA 10, HEPES 10, adjusted to pH 7.25 with KOH. The bath (extracellular) solution was normal Tyrode's solution containing (in mM): NaCl 143, KCl 5.4, NaH_2PO_4 0.33, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 5.0 and glucose 16.6, adjusted to pH 7.4 with NaOH. Recording electrodes were pulled from borosilicate capillaries (Harvard Apparatus, GC150T) using Narishige PP-83 puller (Narishige Scientific Inst., Tokyo, JA) and final resistance was 2.3～2.8 MΩ when filled with pipette solution.

Hypoxic preconditioning and LDH release measurements in vitro

Heart slices were prepared as described previously [10]. Briefly, heart slices 0.4～0.5 mm thick were prepared by cross-sectioning with a Stadie-Riggs microtome (Thomas Scientific, Swedesboro, NJ). LDH was measured in the supernatant and incubation medium using an LDH assay kit (Asan Pharm, Kyunggee-do, Korea). Final values were expressed as percentages of the total LDH released from each ventricular slice. Hypoxic Normal Tyrode's (NT) solution (140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM HEPES, and 16.6 mM glucose adjusted to pH 7.4 with NaOH) was nitrogenated using glucose-free Tyrode's solution, and the NT solution was oxygenated for 45 min prior to use.

Experiment 1. Hypoxia-induced changes in LDH release

Heart slices from each ventricle were bathed in NT solution. After 1 h of bathing in NT solution, serial 1 h periods of hypoxia were induced in each ventricle by replacing the initial NT solution with hypoxic NT solution. After 1 h, we began serial measurements of LDH release, (three times per hour).

Experiment 2. Hypoxia-induced changes in LDH release with preconditioning

Prior to 3 hours of hypoxia, we induced 15 min of hypoxic preconditioning using hypoxic NT solution followed by 15 min of reoxygenation. Other conditions were the same as in the protocol described for Experiment 1.

Experiment 3. Response to glibenclamide

We added 10 μM glibenclamide to the hypoxic NT solution before bathing each ventricle slice. Other conditions were the same as in the protocol described for Experiment 2.

Statistical analysis

Origin 6.0 software (Microcal Software, Inc., Northam-

pton, MA) was used for data analyzing. All data are presented as mean±SEM. Statistical analyses were performed by student's *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Ockir6.2 mRNA expression in the left and right ventricles

To determine the regional expressions of OcKir6.2 in the left and right ventricles, we examined the expression of OcKir6.2 mRNA levels by *in situ* hybridization. The microscopic results of *in situ* hybridization suggested that the optical density of OcKir6.2-positive cells was greater in the right ventricle (a: left ventricle; b: right ventricle; Fig. 1A). The optical density per area (Fig. 1B) indicated that OcKir6.2 mRNA expression was higher in the right ventricle than in the left ventricle.

K_{ATP} current activity in the left and right ventricular myocytes

Whole-cell patch clamping was performed to compare K_{ATP} channel activity between left and right ventricular myocytes. K_{ATP} current was activated via specific opener, 10 μM of pinacidil and reversed by glibenclamide (10 μM, data not shown). Shapes of each ventricular whole-cell current were changed to linear and reached to nano-ampere current level (Fig. 2A). Current-voltage relationship of each ventricular cell was obtained via subtraction of pinadil treated current and control. Current density of right ventricle was higher than left ventricle at 0 mV (104.7 and 76.3 pA/pF, respectively) (Fig. 2B and C). K_{ATP} channel activity in single cell was also higher in right ventricle than left under our experimental condition.

Differences in responses of each ventricle to hypoxic preconditioning

Experiments 1 and 2 were performed to determine whether there are differences in responses to hypoxia and hypoxic preconditioning between the right and left ventricles. LDH release in the left ventricle increased gradually for 3 hours. The right ventricle also showed gradual release of LDH for 3 hours. As LDH release is generally taken to be an indicator of irreversible lethal cell injury, the left ventricle showed greater hypoxic damage than the right ventricle (LDH release in the left and right ventricles,

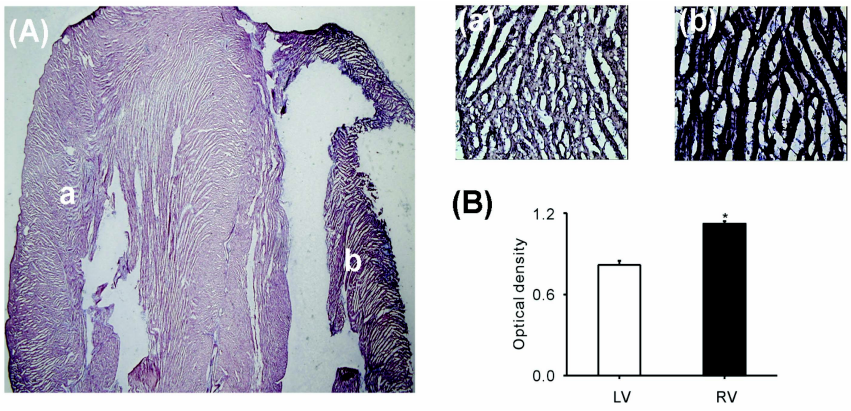

Fig. 1. mRNA expression of OcKir6.2 in rabbit ventricles. (A) The level of OcKir6.2 expression detected by *in situ* hybridization was greater in the right ventricle (b) than that in the left ventricle (a). (B) The optical density calculated using ImageJ software was higher in the right than the left ventricle (0.78 *vs.* 10.5, respectively, n=6, $^*p < 0.05$).

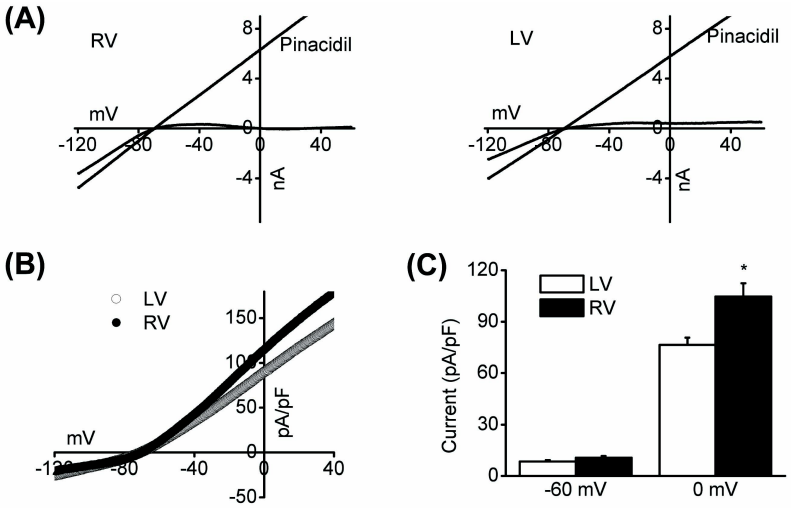

Fig. 2. Whole-cell K_{ATP} channel activity was recorded via ramp pulse (from −120 mV to +60 mV, 0.5 dV/dt) from right and left ventricular myocytes. Representative currents (A) and I-V relationship (B) from right and left myocytes. Application of pinacidil activated K_{ATP} channels. (C) Current density at 0 mV was bigger in right than left myocytes (104.7 and 76.3 pA/pF, respectively, n=7, $^*p < 0.05$).

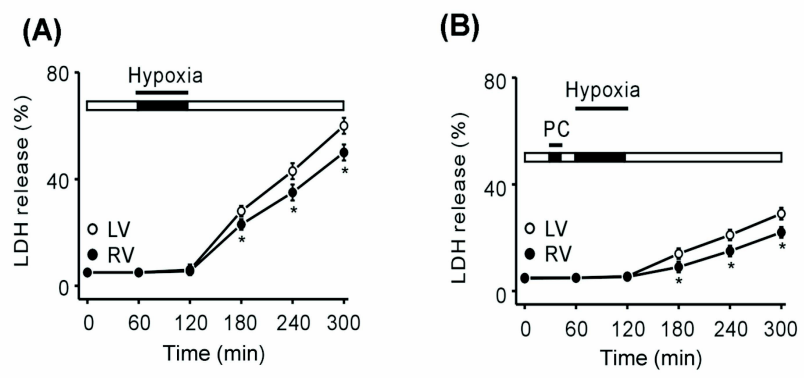

Fig. 3. LDH release in the left and right ventricles under conditions of hypoxia and hypoxic preconditioning. (A) Measurement of LDH release under hypoxic conditions from 1 to 5 h. LDH release in the left ventricle was higher than that in the right ventricle (58.2% *vs.* 49.1%, respectively, n=6; *p<0.05). (B) Measurement of LDH release in hypoxia after hypoxic preconditioning from 1 to 5 h. Left ventricle LDH release was higher than that in the right ventricle (27.3% and 20%, respectively, n=6; *p<0.05). LV (left ventricle, ○), RV (right ventricle, ●), PC, hypoxic preconditioning.

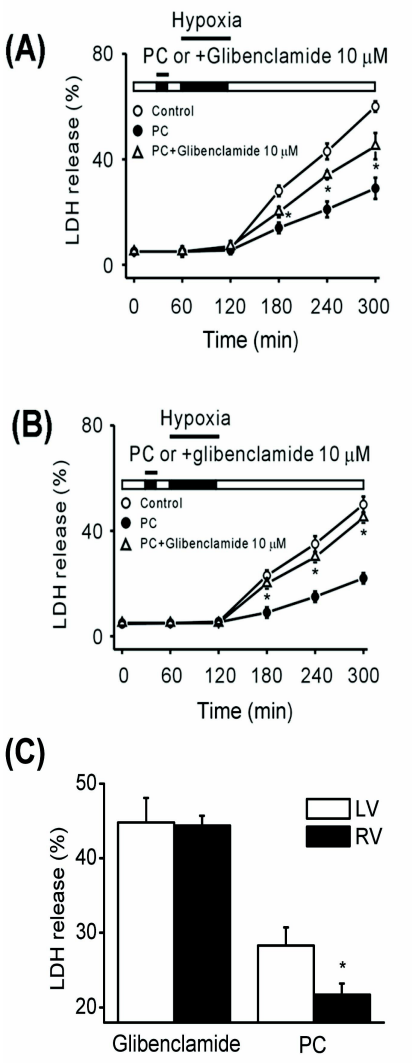

Fig. 4. LDH release in response to glibenclamide treatment in each ventricle. Differences in LDH release between PC (●) and PC+ glibenclamide (△) were greater in the right ventricle (B) than in the left ventricle (A) (n=6, *p<0.05). (C) Glibenclamide sensitivity in the left and right ventricles. LDH release from the left ventricle was greater under PC conditions. However, with addition of glibenclamide, LDH release was not significantly different between the left and right ventricles (n=6, *p<0.05). PC, hypoxic preconditioning.

58.2% and 49.1%, respectively) (Fig. 3A). In Experiment 2, LDH release was significantly decreased by hypoxic preconditioning in both ventricles (LDH release in the left and right ventricles, 27.3% and 20%, respectively) (Fig. 3B).

Different responses to glibenclamide in each ventricle

As describe above, the expression of OcKir6.2 were clearly different between the left and right ventricular tissues. Kir6.2 is thought to be a structural pore and functional subunit of the K_{ATP} channel. During reperfusion injury, activation of K_{ATP} channels functions to protect heart tissues. Addition of the universal K_{ATP} channel blocker glibenclamide after ischemia can abolish the protective effects of K_{ATP} channels. LDH release in the preconditioned control groups was 28.3% for the left ventricle and 21.7% for the right ventricle. However, the levels of LDH release from the left and right ventricles after glibenclamide treatment were almost identical (44.8 and 44.4%, respectively) (Fig. 4A and B). These results indicated that heart tissue damage after ischemia is K_{ATP} channel-dependent and the left and right ventricles show different protective responses. The right ventricle is higher sensitivity to glibenclamide of right ventricle (Fig. 4C), suggesting that K_{ATP} channels play a significant role in preventing hypoxic reperfusion injury to the right ventricle.

DISCUSSION

The results of the present study indicated that the OcKir6.2 gene is expressed in the rabbit heart. The level of OcKir6.2 expression and K_{ATP} current density are higher in the right ventricle than the left ventricle. Left ventricle shows greater hypoxic damage than the right ventricle. Right ventricle is more sensitive to glibenclamide.

In general, myocardial ischemia decreases contractile function of the heart, which induces reduction of the ejection fraction and cardiac output. Moreover, myocardial ischemia induces cardiac chamber dilation with increasing ventricular filling pressure, eventually causes biventricular failure resulting in pulmonary edema or systemic hypotension [11]. In most patients, cardiogenic shock is caused by severe left ventricular dysfunction with left ventricular pump failure. Shock due to predominant right ventricular infarction with right ventricular pump failure is only observed in a small percentage of patients [12]. In-hospital mortality rate of patients were lower with shock due to predominant right ven-

tricular infarction than due to LV pump failure [13].

Right ventricular involvement in myocardial infarction has attracted less attention and protection of the right ventricle has mostly been neglected [11,14]. However, right ventricular involvement in myocardial infarction is known to increase the risk of death, shock and arrhythmia in patient with inferior myocardial infarction [15]. Right ventricle also plays a significant role in myocardial infarction-associated mortality. In addition, recovery of ventricular function after expose to ischemic condition is better in right ventricle comparing with left ventricle. Moreover, oxygen demand of the right ventricle is much more favorable than that of the left ventricle. It suggested that the right ventricle is more durable than the left ventricle under conditions of ischemia and reperfusion [16,17].

A number of studies have suggested the possibility of differences in ischemic resistance between the right and left ventricles. Our results also indicated similar differences in resistance to ischemic injury between the right and left ventricles. The level of LDH release was lower in right ventricle slices than in those of the left ventricle after expose to hypoxic conditions and hypoxic preconditioning (Fig. 3). This effect was abolished by application of K_{ATP} blocker, glibenclamide (Fig. 4). We hypothesized that K_{ATP} channels may be involved in this ischemic resistance, since activation of membrane K_{ATP} channels have been suggested as an endogenous cardioprotective mechanism [18]. Under ischemic conditions, the action potential duration is shortened due to activation of sarcolemmal K_{ATP} channels. The shortening of action potentials is expected to reduce the time for Ca^{2+} influx through L-type Ca^{2+} channels and increase the time for Ca^{2+} efflux through the Na^{+}-Ca^{2+} exchanger. This hypothesis, which suggests that K_{ATP} channels play a key cardioprotective role, has been confirmed in several studies. Activation of sarcolemmal K_{ATP} channels plays an important role in IPC and associated protection of the heart [2-4,19,20]. IPC can be mimicked by KCOs such as pinacidil, and is abolished by the K_{ATP} channel blocker, glibenclamide. Indeed, IPC does not produce significant cardioprotective effects in Kir6.2-deficient mice in comparison with wild-type controls [21]. This report suggested that expression of the K_{ATP} channel in the heart might be related to myocardial damage or protection under ischemic condition. Therefore, we cloned the Kir6.2 gene from rabbits and confirmed its expression in different organs, including the heart (Fig. 1s). We also evaluated the differences in level of OcKir6.2 mRNA expression between the right and left ventricle by *in situ* hybridization analysis (Fig. 1). The optical density of the right ventricle was significantly higher than that of the left ventricle. Moreover, electrophysiological analysis K_{ATP} channel activity was similar to mRNA expression of OcKir6.2. The K_{ATP} channel activity of right ventricle was higher at 0 mV, although it did not show significance at 60 mV. Results of measuring LDH release of each ventricle indicated that glibenclamide sensitivity of the right ventricle was higher than that of the left ventricle (Fig. 4C). As OcKir6.2 expression was higher in the right ventricle, we estimated that the differences in hypoxic response between the two ventricles may be due to differences in expression of K_{ATP} channels.

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