Role of Intracellular Calcium and Cyclic Nucleotides in Realization of Cardioprotective Effects of δ_1- and κ_1-Opioid Receptor Agonists

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 12, pp. 636-639, December, 2009
Original article submitted March 31, 2009

The role of cyclic nucleotides (cAMP, cGMP) and Ca^{2+}-ATPase of the sarcoplasmic reticulum in the mechanism of cardioprotective effects of selective δ_1- and κ_1-opioid receptor agonists DPDPE and U-50488 was studied under conditions of global ischemia and reperfusion of isolated and perfused rat heart. Activation of both types of opioid receptors 2-fold reduced the reperfusion release of creatine phosphokinase. The cardioprotective effect of U-50488 was paralleled by a 2-fold decrease in cAMP content in the myocardium, while DPDPE did not modify the content of cAMP throughout the experiment. None of these substances changed the content of cGMP in the myocardium. The cardioprotective effect of DPDPE was not observed after inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase with cyclopiazonic acid. The cardioprotective effect of U-50488 was associated with reduction of cAMP level in the myocardium, while the cytoprotective effect of DPDPE was mediated by opioidergic modulation of Ca^{2+} transport at the level of the sarcoplasmic reticulum.

Key Words: *cyclic nucleotides; opioid receptors; sarcoplasmatic reticulum; ischemia; reperfusion*

Opioid receptors (OR) are involved in the formation of heart resistance to ischemia and reperfusion [1-3,10]. Preventive treatment with OR agonists reduces the number of irreversibly damaged cardiomyocytes in myocardial infarction [10]. However, the role of intracellular signal systems (cAMP, cGMP, inositol triphosphate, Ca^{2+}, *etc.*) mediating the cardioprotective effects of opioids remains not quite clear.

It is known that opioids are involved in the regulation of Ca^{2+} transport at the level of the sarcoplasmic reticulum (CPR) in intact cardiomyocytes [11]. Interactions between OR and intracellular Ca^{2+} under conditions of ischemia/reperfusion of the myocardium remains not studied. cAMP plays an important role in the regulation of Ca^{2+} transport in heart cells; its excess, similarly as the increase of Ca^{2+} concentration in the cardiomyocyte myoplasm, causes irreversible damage to heart cells [11]. Stimulation of OR can lead to inhibition of adenylate cyclase and reduction of cAMP level [7]. However, cAMP is not the only intracellular messenger involved in the formation of heart resistance to ischemia and reperfusion. Myocardial tolerance of reperfusion provides stimulation of NO production and cGMP synthesis [9]. We hypothesized that the protective effects of opioids can result from intensification of cGMP synthesis in the myocardium.

We studied the role of cAMP, cGMP, and Ca^{2+} of SPR in the realization of the cardioprotective effects of δ_1- and κ_1-OR agonists *in vitro*.

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0007-4888/09/1486-0877

MATERIALS AND METHODS

Experiments were carried out on isolated hearts from male Wistar rats (250-300 g) narcotized with ethyl ether. The hearts were removed from the thorax, rapidly transferred into a flask with cold (4°C) Krebs—Henseleit solution, and left there until arrest of spontaneous contractions. Langendorff perfusion of the heart with Krebs—Henseleit solution was carried out through a cannula inserted into the ascending aortic arch. Oxygenated perfusion solution (37°C, pH 7.4) contained (in mM): 120 NaCl, 4.8 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 20.0 $NaHCO_3$, and 10.0 glucose (all reagents were from ICN Biomedicals). The perfusion was stopped for 45 min (total normothermal ischemia of the myocardium) and then resumed; the observation was continued for 30 min.

Stimulation of δ_1-OR was performed with H-Tyr-D-Pen-Gly-Phe-D-Pen-OH selective δ_1 agonist (DPDPE; Multiple Peptide Systems) [6]. The κ_1-OR were stimulated with trans(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide HCl selective κ_1 agonist (U-50488; Tocris Cookson) [6]. After 20-min adaptation to normoxic perfusion and 20 min before the start of ischemia/reperfusion, one of these agonists was added to Krebs–Henseleit solution in a final concentration of 0.1 μmol/liter. After 10-min perfusion with the preparation, the heart was washed (10 min) from the ligand. The duration of the next total ischemia was 45 min, reperfusion 30 min. The substances were dissolved in saline directly before the experiment. Isolated hearts subjected to 45-min total ischemia and 30-min reperfusion after 40 min of stabilization period served as the control.

The role of SPR Ca^{2+}-ATPase was evaluated by specific inhibition of this enzyme with cyclopiazonic acid (CPA) in a final concentration of 100 nmol/liter [5]. This reagent was dissolved in DMSO and then diluted in Krebs–Henseleit solution, DMSO concentration in the solution ≤0.01% [5]. The protocol of the experiments with CPA was similar to that used in studies of the effects of DPDPE and U-50488.

The content of cAMP and cGMP in myocardial tissue in different periods of the experiment was measured using standard commercial radioimmunoassay kits RIA AMPc/cAMP and RIA cGMP, respectively (Immunotech). The hearts for measurements were frozen and stored in liquid nitrogen until cAMP and cGMP assay. Radioactivity of the samples was measured on a Gamma-12 gamma scintillation counter (Russia).

The results were statistically processed using Mann—Whitney test.

RESULTS

Exposure of hearts from control rats to ischemia and reperfusion led to the development of irreversible damage to cardiomyocyte membranes. This was indirectly proven by a 4-fold increase in creatine kinase activity in the solution flowing from the heart during reperfusion compared to the preischemic level (Fig. 1). After stimulation of δ_1- or κ_1-OR with agonists, the severity of ischemic and reperfusion injuries of the myocardium significantly decreased: reperfusion release of creatine kinase in the solution flowing from the heart decreased by 45 and 40%, respectively, in comparison with the control (Fig. 1). Similar results were reported by other investigators. For example, *in vivo* eperiments showed that intravenous injection of selective κ-agonists reduced the size of the reperfusion necrotic zone in rat myocardium [10], while stimulation of κ-OR on membranes of isolated cardiomyocytes reduced the severity of anoxia/reoxygenation damage to these cells [13]. On the other hand, experiments

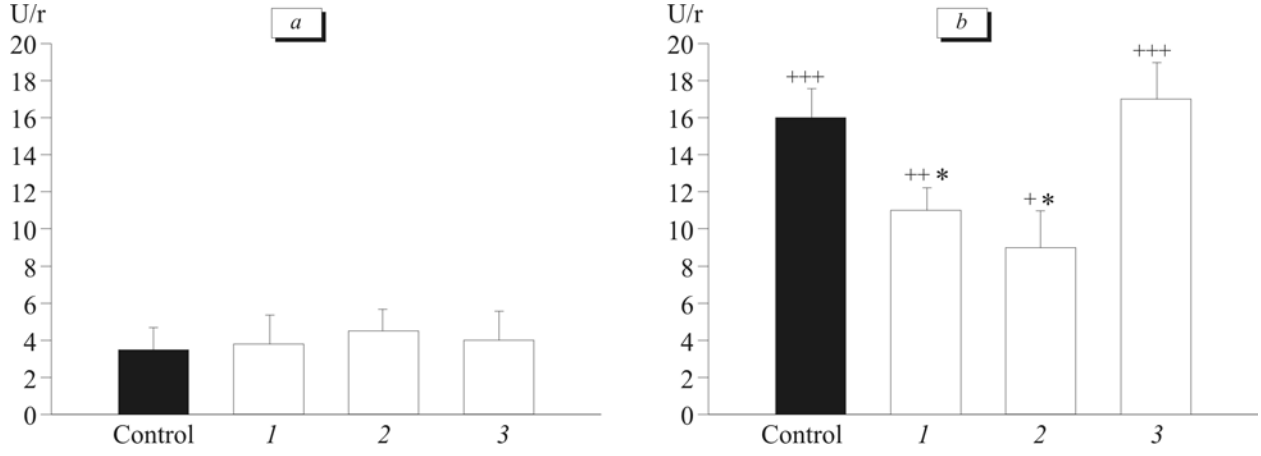

Fig. 1. Creatine phosphokinase activity in perfusion solution after preactivation of κ_1- and δ_1-OR with DPDPE (*1*), U-50488 (*2*), and combination of CPA and DPDPE (*3*). *a*) before ischemia; *b*) during reperfusion. **p*<0.05 compared to the control; ^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001 compared to the level before ischemia.

on isolated perfused rat hearts showed that addition of bremazocine (κ_2-OR agonist) to the perfusion solution caused enlargement of the necrotic zone in the myocardium [3]. It is noteworthy that the authors using κ_1-receptor agonists observed an increase in heart resistance to ischemia/reperfusion [10,13], while investigators using bremazocin *in vitro* observed aggravation of the ischemic and reperfusion damage to the heart [3]. These contradictions can be explained by the presence of two κ-OR subtypes on cardiomyocyte membranes [14]. We detected a cytoprotective effect of U-50488 κ_1-agonist in a concentration of 0.1 μmol/liter, providing the interaction of this ligand with only κ_1-OR [6]. The inhibitory effect of κ-agonists on adenylate cyclase activity [7] and hence, on cAMP synthesis suggests that κ_1-opioidergic reduction of cAMP production in the myocardium during ischemia and reperfusion plays the key role in the realization of the cardioprotective effect of U-50488.

The content of cAMP in the myocardium after 30-min reperfusion decreased almost 2-fold in response to κ_1-OR stimulation and did not change after application of δ_1-agonist (Fig. 2, *a*).

Cyclic AMP is not the only intracellular messenger, which can participate in the formation of heart resistance to injuries caused by ischemia and reperfusion. There is evidence on the key role of NO in myocardial resistance to reperfusion [9]; it stimulates guanylate cyclase and cGMP synthesis. In addition, morphine stimulates NO production in isolated human endotheliocytes and heart preparations [12]. We hypothesized that the cardioprotective effect of opioids can result from more intensive synthesis of NO and cGMP in the myocardium. We previously showed that intravenous dalargin (opioid peptide) injected 15 min before coronary occlusion increased cGMP level in the ischemic focus and intact myocardium *in vivo* [2].

The level of cGMP in the myocardial tissue was measured on the 10th minute of perfusion of isolated heart with solution containing δ- and κ-OR agonists. The level of cGMP was measured under the same conditions at the end of the 30th minute of reperfusion (Fig. 2, *b*). However, experiments on isolated hearts revealed no changes in cGMP level in the myocardium in response to stimulation of δ- or κ-OR (Fig. 2, *b*). It seems that the role of cGMP in the mechanism of the cardioprotective effect of opioids requires further research.

Hence, the cytoprotective effect observed after stimulation of cardiac δ_1-OR was not associated with changes in intracellular levels of cAMP and cGMP. We hypothesized that it was caused by the opioidergic changes in Ca^{2+} transport in cardiomyocytes. This hypothesis is supported by our previous data indicating that DPDPE increased the end-diastolic pressure before ischemia [1]. These changes in the diastolic function of the heart are usually attributed to an increase in Ca^{2+} concentration in the cytoplasm. Moreover, stimulation of OR promotes intense mobilization of Ca^{2+} from SPR. We hypothesized that the cardioprotective effect of DPDPE could be caused by changes in Ca^{2+} transport from SPR to the myoplasm and carried out experiments with CPA. Pretreatment with CPA completely abolished the cardioprotective effect observed as a result of δ_1-OR stimulation (Fig. 1). Hence, modulation of Ca^{2+} transport at the SPR level seems to play the key role in the mechanism of δ_1-receptor-mediated cardiac protection during ischemia/reperfusion.

However, excessive accumulation of Ca^{2+} in the cytoplasm might involve cardiomyocyte injury during ischemia/reperfusion by the mechanism resembling the "calcium paradox" [12], which was not observed. By contrast, heart tolerance to ischemia/reperfusion increased under conditions of δ_1-OR stimulation.

Two explanations of this phenomenon can be proposed. One of them is as follows: OR stimulation pro-

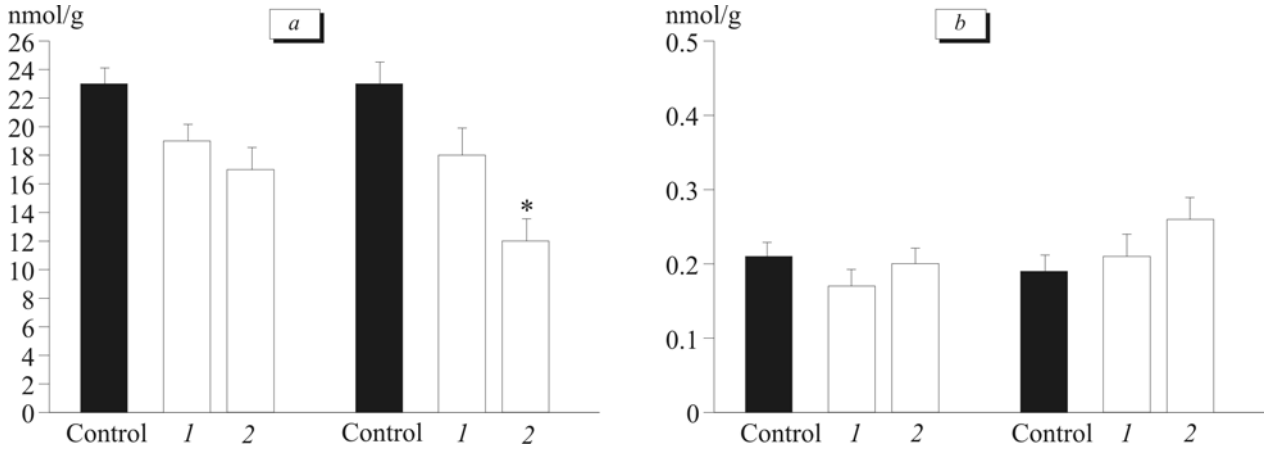

Fig. 2. Levels of cAMP (*a*) and cGMP (*b*) in the myocardium after preliminary stimulation of κ_1- and δ_1-OR with DPDPE (*1*) and U-50488 (*2*). *$p<0.05$ compared to the control.

motes intensive mobilization of Ca^{2+} from the SPR [11] with subsequent exhaustion of Ca^{2+} depots in SPR during the preischemic period. These changes in calcium homeostasis can prevent Ca^{2+} overload of cardiomyocytes during ischemia and reperfusion [5] and hence, improve heart resistance to hypoxia and reoxygenation.

Another possible mechanism of δ_1-opioidergic increase of myocardial resistance to the pathogenic effects of ischemia and reperfusion can be based on activation of protein kinase C catalyzing phosphorylation of proteins responsible for heart resistance to ischemic and reperfusion exposure [4]. It is noteworthy that activity of protein kinase C can increase in response to short-term elevation of $[Ca^{2+}]_i$ [8]. We found that inhibition of Ca^{2+}-ATPase with CPA abolished elevation of end-diastolic pressure in response to δ_1-OR stimulation [2] and the cardioprotective effect of DPDPE. Treatment with CPA promoted exhaustion of Ca^{2+} stores in SPR [5] with subsequent reduction of $[Ca^{2+}]_i$, and we observed a decrease in heart contraction amplitude in response to addition of CPA into perfusate, which could be explained by reduction of Ca^{2+} release from SPR.

Hence, our findings persuasively prove the involvement of cardiac δ_1- and κ_1-OR in the formation of myocardial resistance to the destructive effects of ischemia and reperfusion. Stimulation of these receptors involves inhibition of cardiomyocyte damage during ischemia/reperfusion. The cardioprotective effect of U-50488 can be explained by reduction of cAMP level in the myocardium, while similar effect of DPDPE is mediated through opioidergic modification of Ca^{2+} transport at the SPR level.

The study was supported by Ministry of Education and Science of the Russian Federation (grant No. 2.1.1/210).

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