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A novel cardioprotective function of adenosine A₁ and A₃ receptors during prolonged simulated ischemia

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Abstract

The possible cardioprotective roles of adenosine A₁ and A₃ receptors were investigated in a cardiac myocyte model of injury. The adenosine A₃ receptor is a novel cardiac receptor capable of mediating potentially important cardioprotective functions. Prolonged hypoxia with glucose deprivation was used to simulate ischemia and to induce injury in cardiac ventricular myocytes cultured from chick embryos 14 days in ovo. When present during the prolonged hypoxia, the adenosine A₃ agonists *N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) and 2-chloro- *N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (CI-IB-MECA) caused a dose-dependent reduction in the extent of hypoxia-induced injury as manifested by a decrease in the amount of creatine kinase released and the percentage of myocytes killed. The adenosine A₁ agonists 2-chloro-*N*⁶-cyclopentyladenosine (CCPA), *N*⁶-cyclohexyladenosine, and adenosine amine congener were also able to cause a decrease in the extent of myocyte injury. The A₁ receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine blocked the cardioprotective effect of the A₁ but not of the A₃ agonists. Conversely, the selective A₃ antagonists MRS-1191 and MRS-1097 blocked the protection induced by CI-IB-MECA but had minimal effect on that caused by CCPA. Thus the cardioprotective effects of A₁ and A₃ agonists were mediated by their respective receptors. This study defines a novel cardioprotective function of the cardiac A₃ receptor and provides conclusive evidence that activation of both A₁ and A₃ receptors during hypoxia can attenuate myocyte injury.

Keywords

cardioprotection; heart cell; purines

ADENOSINE IS RELEASED in large amounts during myocardial ischemia and can mediate potentially important protective functions in the cardiovascular system (1, 4–6, 9, 14, 17–19, 25). Previous studies have shown that adenosine-receptor agonists can

precondition the heart when given before ischemia (4, 5, 9, 14, 17, 18) and can cause a reduction in infarct size or improvement in left ventricular function when given during reperfusion (1, 19) or during both low-flow ischemia and reperfusion in the isolated perfused heart (7, 21, 22). Although activation of adenosine A₁ and A₃ receptors has been shown to mimic the cardioprotective effect of preconditioning (3, 10, 23, 24), their roles in mediating the protective effect of adenosine administered during ischemia or during reperfusion are not known. Furthermore, the cardioprotective effect of exogenous adenosine infused during ischemia in the intact heart may be exerted at the level of the coronary vasculature, circulating neutrophils, or cardiac myocytes. It is not known whether activation of myocyte adenosine receptors during ischemia can cause cardioprotection. Previous studies by Liang (16) and Strickler et al. (23) characterized a cardiac myocyte model of injury, which is induced by exposure of myocytes to prolonged hypoxia in glucose-free media. The objectives of the present study were to determine whether activation of adenosine receptors during prolonged hypoxia can attenuate the hypoxia-induced myocyte injury and to investigate the role of A₁ and A₃ receptors in mediating the adenosine-induced cardiac myocyte protection.

METHODS

Preparation of cultured ventricular cells

Ventricular cells were cultured from chick embryos 14 days in ovo as previously described (16, 23). The cells were cultivated in a humidified 5% CO₂-95% air mixture at 37°C. All experiments were performed on *day 3* in culture, at which time the cells exhibited rhythmic spontaneous contraction. The medium was changed to a *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered medium containing (in mM) 139 NaCl, 4.7 KCl, 0.5 MgCl₂, 0.9 CaCl₂, and 5 HEPES and 2% fetal bovine serum, pH 7.4, at 37°C before the myocytes were exposed to the various conditions at 37°C. Control myocytes were maintained in the HEPES-buffered medium under room air. A 90-min exposure of the myocytes to hypoxia with glucose deprivation was used to induce cell injury. Hypoxia was produced by placing the cells in a hypoxic incubator (NuAire) where O₂ was replaced by N₂ as previously described (16, 23). The effects of adenosine-receptor agonists and antagonists on the extent of myocyte injury were determined by exposure of the cells to these agents during prolonged hypoxia.

Determination of cell injury

The determination of myocyte injury was made at the end of the 90-min hypoxic period, at which time the myocytes were taken out of the hypoxic incubator and reexposed to room air (normal percentage of O₂). Aliquots of the medium were then obtained for creatine kinase (CK) activity measurement, which was followed by quantitation of the number of viable cells. Measurement of the basal level of cell injury was made after parallel incubation of control cells under a normal percentage of O₂. The extent of hypoxia-induced injury to the ventricular cell was quantitatively determined by the percentages of cells killed and by the amount of CK released into the medium according to a previously described method (16, 23). Prior studies demonstrated that the cell viability assay distinguished the hypoxia-damaged from the control normoxia-exposed cells (16, 23). In brief, the medium was

replaced with a trypsin-EDTA buffer to detach the cells, which was then followed by sedimentation of the viable myocytes. Parallel changes in the percentage of cells killed and CK released (16, 23) further validated this assay for the percentage of cells killed. The amount of CK was measured as enzyme activity (in units per milligram), and the increase in CK activity above the control level was determined. The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group divided by the number of cells in the control group multiplied by 100%.

Synthesis of adenosine A₁ and A₃ receptor-selective agents

*N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA), 2-chloro-*N*⁶-(3-iodobenzyl)adenosine-5'-*A*-methyluronamide (Cl-IB-MECA), and adenosine amine congener (ADAC) were synthesized as described previously (8, 11, 13). 3,5-Diethyl 2-methyl-6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1097) and 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191) were synthesized as described previously (12).

Materials

The adenosine analogs 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-chloro-*N*⁶-cyclopentyladenosine (CCPA), and *N*⁶-cyclohexyladenosine (CHA) were from Research Biochemicals International (Natick, MA). Embryonic chick eggs were from Spafas (Storrs, CT).

RESULTS

Exogenous adenosine causes a decrease in the extent of cardiac myocyte injury during prolonged hypoxia

Prolonged exposure to hypoxia in glucose-free medium induced significant cardiac myocyte injury, with a large increase in the release of CK [28.5 ± 1.5 (SE) units/mg; $n = 4$] and in the percentage of cells killed ($30 \pm 2\%$; $n = 4$). Adenosine ($10 \mu\text{M}$), when added to the medium during the 90-min hypoxic period, caused a decrease in the amount of CK released (14.9 ± 3 units/mg; $n = 3$) and in the percentage of cells killed ($12 \pm 2\%$; $n = 4$). This effect of exogenous adenosine was blocked by the nonselective adenosine-receptor antagonist 8-sulfophenyltheophylline (8-SPT; $100 \mu\text{M}$; in the presence of adenosine and 8-SPT, CK released = 31 ± 5 units/mg and cells killed = $28 \pm 3\%$; $n = 5$). The presence of 8-SPT during hypoxia had no effect on the level of myocyte injury (CK released = 29.6 ± 1.2 units/mg; cells killed = $31.6 \pm 1.5\%$; $n = 3$). These data suggest that activation of adenosine receptors during prolonged hypoxia can protect the myocyte against injury. Because adenosine can activate both the cardiac A₁ and A₃ receptors and because 8-SPT ($100 \mu\text{M}$) can block both receptors (23), the data are consistent with the notion that either receptor or both receptor subtypes can mediate the cardioprotective function of adenosine. This question was examined next with selective agonists and antagonists.

Cardioprotective effect of adenosine A₃ agonist during prolonged hypoxia

To examine whether activation of adenosine A₃ receptors is capable of attenuating myocyte injury during prolonged hypoxia, agonists selective at the A₃ receptor were used. A prior study (23) demonstrated that both IB-MECA and CI-IB-MECA are highly selective at the chick cardiac A₃ receptor. Either A₃ agonist when present during prolonged hypoxia was capable of protecting the cardiac myocytes against hypoxia-induced injury (Fig. 1). The cardioprotective effect of A₃ agonists was quantitated as a decrease in the amount of CK released and the percentage of myocytes killed [statistically significant at 1 and 10 nM CI-IB-MECA, $P < 0.01$ by analysis of variance (ANOVA) and t -test]. The presence of the A₁ receptor-selective antagonist DPCPX had no effect on the ability of IB-MECA or CI-IB-MECA to mediate their cardioprotective effects; the A₃ agonist-induced decreases in the percentage of cells killed and CK released were similar in the presence and absence of 1 μ M DPCPX (data not shown). These data indicate that the cardioprotective effect of the A₃ agonists was not due to activation of the cardiac A₁ receptor.

To prove that the A₃ agonist-induced cardioprotection is mediated by the A₃ receptor, antagonists selective at the A₃ receptor were employed. Figure 2 demonstrates that the A₃ receptor-selective antagonist MRS-1191 blocked the CI-IB-MECA-induced cardioprotection. The levels of CK released and percentages of cells killed were significantly higher in myocytes exposed to CI-IB-MECA and 30 nM, 300 nM, and 3 μ M MRS-1191 ($P < 0.01$ by ANOVA and t -test). Another A₃-receptor antagonist, MRS-1097, was also able to block the cardioprotective effect of CI-IB-MECA (data not shown). These data provide conclusive evidence that activation of the A₃ receptor can produce a potent cardioprotective effect when administered during prolonged hypoxia.

Effect of adenosine A₁-receptor activation on the extent of cardiac myocyte injury

Because the A₁ receptor is also present on the cardiac myocyte, the question arises regarding whether activation of the A₁ receptor can confer a cardioprotective effect during prolonged hypoxia. A prior study (23) showed that CCPA, a known A₁ agonist, is highly selective at the A₁ receptor on these cardiac myocytes. CCPA caused a dose-dependent reduction in the percentage of myocytes killed (Fig. 3) and in the amount of CK released (data not shown; $P < 0.01$ by ANOVA and t -test). Two other A₁ receptor-selective agonists, ADAC and CHA, were also able to protect the myocytes when present during prolonged hypoxia. The cardioprotection stimulated by CCPA, CHA, and ADAC was blocked by the A₁ antagonist DPCPX. On the other hand, neither MRS-1191 nor MRS-1097 was able to block the CCPA-induced cardioprotection (Fig. 2), providing definitive evidence that the A₁-agonist effect is mediated by the A₁ receptor.

Although the number of viable cells was determined quickly after reexposure of cardiac myocytes to a normal percentage of O₂ (reoxygenation), the A₁ or A₃ agonist was nevertheless briefly present before replacement with the trypsin-EDTA buffer for cell viability assay. Thus it is possible that the decrease in myocyte injury is due to the protection against a reoxygenation injury. To study this possibility, CCPA or CI-IB-MECA was added immediately on reoxygenation after the 90-min hypoxic exposure. CCPA or CI-IB-MECA was maintained in the medium for an additional hour before determination of the percentage

of myocytes killed. Although CCPA or CI-IB-MECA was able to protect the myocytes when it was present during the reoxygenation, the extent of protection was small (myocytes killed after the 90-min hypoxic period, $26.5 \pm 1.0\%$, $n = 6$, vs. with CCPA present, $22.1 \pm 1.5\%$, $n = 5$, or vs. with CI-IB-MECA present, $23.0 \pm 1.4\%$, $n = 5$; $P < 0.05$ by ANOVA and t -test).

DISCUSSION

Adenosine can exert two principal cardioprotective effects. Adenosine can precondition the heart with a reduction in the size of myocardial infarction (4, 5, 9, 14, 17, 18). Intracoronary administration of adenosine during reperfusion after prolonged no-flow ischemia can also limit infarct size in the intact heart (1, 19). Although several studies (7, 21, 22) demonstrated an enhanced recovery of ventricular function when adenosine was infused during low-flow ischemia and reperfusion, questions arise regarding whether the beneficial effect of adenosine 1) is exerted during the ischemia or during the reperfusion, 2) is due to activation of adenosine receptors because the adenosine concentration used was high (50–100 μM), and 3) is mediated at the level of the coronary vasculature, circulating neutrophils, or cardiac myocytes. Previous studies by Liang (16) and Strickler et al. (23) characterized a cardiac myocyte model of injury, which is induced by the exposure of myocytes to a prolonged period of hypoxia in glucose-free medium. The objectives of the present study were to investigate whether the presence of exogenous adenosine during hypoxia and glucose deprivation can attenuate myocyte injury and to study the role of adenosine receptor subtypes in mediating the decrease in myocyte injury.

The addition of exogenous adenosine during exposure of myocytes to prolonged hypoxia resulted in a significant decrease in the extent of myocyte injury. The concomitant presence of the adenosine-receptor antagonist 8-SPT reversed the protective effect of adenosine. Although an adenosine-induced vasodilatation and neutrophil inhibition may contribute to the cardioprotective effect of adenosine in vivo, the present data suggest that activation of adenosine receptors on the cardiac myocyte during prolonged hypoxia is capable of protecting the myocyte against injury. The presence of adenosine in the medium during the hypoxic exposure is not necessarily equivalent to an intracoronary infusion of adenosine during low-flow ischemia. However, the data suggest that adenosine, acting via adenosine receptors, can achieve cardioprotection during an actual injury-inducing stimulus such as hypoxia with glucose deprivation.

Recent studies have shown that a novel adenosine A_3 receptor is expressed in the heart (26) and is likely present on the cardiac myocyte (23). The expression of cardiac A_3 receptors suggests a novel function for this receptor. The present data indicate that A_3 agonists administered during prolonged hypoxia caused a large decrease in the extent of myocyte injury. This protective effect of A_3 agonists was mediated solely by the adenosine A_3 receptor because the A_3 agonist-induced protection was completely abolished by the A_3 antagonists MRS-1097 and MRS-1191 but was not affected by the A_1 receptor-selective antagonist DPCPX. Together, these findings suggest a novel cardioprotective function of the adenosine A_3 receptor and further support the notion that an adenosine A_3 receptor is present on the cardiac myocyte (23). The potential cardioprotective function of adenosine A_1 receptors, which are expressed on the cardiac myocyte (2, 15, 20), was also investigated. The

presence of the A₁ receptor-selective agonists during prolonged hypoxia resulted in a decrease in the extent of cardiac myocyte injury. Specificity of the coupling of the A₁ receptor to a cardioprotective effect was established by the finding that the A₁ agonist-mediated protection was only blocked by the A₁ antagonist DPCPX. The A₃ antagonists had minimal effect on such protection. Because the A₁ or the A₃ agonist, added just before the 90-min hypoxic period, was present during the reoxygenation, the question arises as to whether the salutary effect is due to protection against a reoxygenation injury. Although the presence of the A₁ or the A₃ agonist during reoxygenation had a protective effect, the extent of such protection was very small compared with that caused by the agonist during the prolonged hypoxia. Thus the cardioprotection caused by the A₁ or the A₃ agonist is due primarily to the effect of A₁ or A₃-receptor activation, respectively, during sustained hypoxia. The molecular mechanism(s) underlying the A₁ and A₃ receptor-mediated cardioprotective function is not well understood. A number of potential mediators, such as phospholipase D, diacylglycerol, protein kinase C, and the ATP-sensitive K⁺ (K_{ATP}) channel, have all been implicated in causing the protective effect of adenosine. Although a protein kinase C-induced activation of the K_{ATP} channel has been speculated to mediate this protective function of adenosine, the exact role of these mediators and their interaction remain unknown. Elucidation of their function deserves further investigation.

Overall, the present study demonstrates a novel protective function of the cardiac A₃ receptor. In addition to the role of both receptors in mediating preconditioning of the cardiac myocyte, the data provide conclusive evidence that activation of both receptors can also attenuate myocyte injury during prolonged hypoxia. Thus agonists selective at the A₁ or the A₃ receptors represent novel potent cardioprotective agents even when hypoxia has already begun. These data should have important clinical implications in the treatment of ischemic heart disease and suggest that A₁ and A₃ receptor-selective agonists may reduce the size of myocardial infarction when given during infarct-producing ischemia.

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Fig. 1.

Effect of adenosine A_3 receptor agonists on hypoxia-induced myocyte injury. Cardiac ventricular myocytes were cultured from chick embryos 14 days in ovo, and myocyte injury was induced as described in METHODS. N^6 -(3-iodobenzyl)adenosine-5'- N -methyluronamide (IB-MECA) and 2-chloro- N^6 -(3-iodobenzyl)adenosine-5'- N -methyluronamide (CI-IB-MECA) were added to medium at concentrations indicated in presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 1 μ M) during hypoxia. Percentage of myocytes injured (open symbols) and amount of creatine kinase (CK; solid symbols) released were determined after hypoxia. Data are means of 4 experiments. At 1 and 10 nM CI-IB-MECA or IB-MECA, levels of CK released and percentages of cells killed were significantly lower than those determined in myocytes exposed to hypoxia only in absence of any adenosine agonist or antagonist [$P < 0.01$ by analysis of variance (ANOVA) and t -test].

Fig. 2.

Effect of adenosine A_3 -receptor antagonists on 2-chloro- N^6 -cyclopentyladenosine (CCPA)- and Cl-IB-MECA-induced cardioprotective effects. Cultured ventricular myocytes were prepared, and extent of hypoxia-induced myocyte injury was determined as described in METHODS. A_3 antagonist MRS-1191 was present at concentrations indicated with CCPA (10 nM) or Cl-IB-MECA (10 nM) during 90-min hypoxic period. Percentage of myocytes killed (A) and amount of CK released (B) were determined after prolonged hypoxia. Data are means $\pm$ SE of 3 experiments. In presence of Cl-IB-MECA and 30 nM, 300 nM, and 3 μ M MRS-1191, levels of CK released and percentages of cells killed were significantly higher than those determined in myocytes exposed to Cl-IB-MECA only ($P < 0.01$ by ANOVA and t -test).

Fig. 3.

Effect of adenosine A₁ agonists on cardiac myocyte injury. Cultured ventricular myocytes were prepared, and extent of hypoxia-mediated myocyte injury was determined as described in METHODS. CHA, *N*⁶-cyclohexyladenosine; ADAC, adenosine amine congener. Adenosine A₁-receptor agonists were added to medium at concentrations indicated in absence or presence of A₁-receptor antagonist DPCPX during prolonged hypoxia. Percentage of cells killed was determined after hypoxic exposure and removal of A₁-receptor agonists and antagonist. Data are means of 4 experiments. At 1 and 10 nM concentrations of A₁ agonists, percentages of myocytes killed were significantly lower than those obtained in presence of either of the 2 A₁-agonist concentrations and DPCPX (1 μ M) ($P < 0.01$ by ANOVA and *t*-test).