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Cardiac myocytes rendered ischemia resistant by expressing the human adenosine A_1 or A_3 receptor

CHARLES DOUGHERTY, **JULIE BARUCHA**, **PETER R. SCHOFIELD**[*], **KENNETH A. JACOBSON**[†], and **BRUCE T. LIANG**[1]

Department of Medicine, Cardiovascular Division, and Department of Pharmacology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104, USA

[*]Garvan Institute for Medical Research, Sydney, Australia

[†]Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892–1008, USA

Abstract

Adenosine is an important mediator of the endogenous defense against ischemia-induced injury in the heart. Adenosine can achieve cardioprotection by mediating the effect of ischemic preconditioning and by protecting against myocyte injury when it is present during the infarct-producing ischemia. A novel adenosine A_3 receptor can mediate this protective function. One approach to achieve cardioprotection is to enhance myocardial sensitivity to the endogenous adenosine by increasing the number of adenosine receptors instead of administering an adenosine receptor agonist. The objective of the present study was to investigate whether genetic manipulation of the cardiac myocyte, achieved by gene transfer and overexpression of the human A_3 receptor cDNA, renders the myocytes resistant to the deleterious effect of ischemia. Prolonged hypoxia with glucose deprivation, causing myocyte injury and adenosine release, was used to simulate ischemia in cultured chick embryo ventricular myocytes. During simulated ischemia, cultured myocytes with enhanced expression of the human A_3 receptor and showed significantly higher ATP content, fewer cells killed, and less creatine kinase released into the medium than either control or mock-transfected myocytes. Also, increased expression of the A_3 receptor caused an enhanced cardioprotective effect by the preconditioning ischemia. Overexpressing the adenosine A_1 receptor also led to increased protection against ischemia-induced myocyte injury as well as an enhanced preconditioning effect. Thus, increasing the receptor level improves the myocyte sensitivity to the endogenous adenosine, which in turn causes all of the cardioprotective effects found for exogenously administered adenosine agonists. The study provides the first proof for the new concept that an increased expression of the human A_3 receptor in the cardiac myocyte can be an important cardioprotective therapeutic approach.

Keywords

heart; purinergic; ventricular myocyte; gene transfer

[1]Correspondence: 504 Johnson Pavilion, University of Pennsylvania, Medical Center, 3610 Hamilton Walk, Philadelphia, PA 19104, USA. liangb@mail.med.upenn.edu.

Adenosine is released during cardiac ischemia and can mediate important protective functions in the heart (1–6). Previous studies have shown that adenosine or adenosine receptor agonists can cause a reduction in infarct size or improvement in left ventricular function when given during reperfusion (1, 6) or during both low-flow ischemia and reperfusion in the isolated, perfused heart (7, 8). The protective effect of adenosine in the intact heart is mediated by adenosine receptors. An enhanced receptor activation achieved, for example, by an increased agonist concentration will result in cardioprotection. Because the reserve of cardiac adenosine receptors is limited (9, 10), another approach is to increase the level of adenosine receptors in the cardiac myocytes and thereby render them more responsive to stimulation by adenosine. Since adenosine release is increased during ischemia, an enhanced receptor-mediated responsiveness to adenosine should lead to an increased cardioprotective effect by the endogenous adenosine. The proof of this concept in the intact heart is in practice difficult because of inefficient gene transfer in the intact myocardium. However, transgenic mouse hearts overexpressing the adenosine A_1 receptor exhibited enhanced resistance to the deleterious effect of ischemia (11), demonstrating that overexpression of one of the adenosine receptor subtypes can lead to increased cardioprotection.

The cardioprotective effect of adenosine in the intact heart is exerted, at least partially, at the level of cardiac myocytes (12, 13). Thus, the development of a cardiac myocyte model of ischemia and injury would facilitate further testing of this concept and determination of the cardioprotective effect after expression of other adenosine receptor subtypes. Our previous studies have characterized a cardiac myocyte model of injury produced by exposure of myocytes to simulated ischemia. Simulated ischemia was induced by prolonged hypoxia and glucose deprivation (12, 13) and was associated with an increased release of adenosine into the media (14). Adenosine can achieve cardioprotection by mediating the effect of ischemic preconditioning and by protecting against myocyte injury when it is present during the infarct-producing ischemia. The most recently characterized subtype of the adenosine receptor family, the A_3 receptor, can mediate this protective function. The objective of the present study was to investigate whether an increased expression of the human A_3 adenosine receptor will sensitize cardiac myocytes to the protective effect of adenosine released during the ischemia. The current study was aimed at testing the hypothesis that genetically altered cardiac myocytes, in which an increased expression of human A_3 receptors is achieved by cDNA transfection, are more resistant to the deleterious effect of ischemia. Parallel studies to examine the effects of overexpressing the human adenosine A_1 receptor were also carried out.

MATERIALS AND METHODS

Preparation of cultured ventricular myocytes and simulation of ischemia

Ventricular cells were cultured from chick embryos 14 days in ovo and were cultivated in a humidified 5% CO_2-95% air mixture at 37°C, as previously described (11, 12, 14). All experiments were performed on day 3 in culture, at which time cells exhibited spontaneous contraction and more than 90% of cultures were myocytes (14). The medium was changed to a HE-PES-buffered medium containing (mM) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$,

5 HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), and 2% fetal bovine serum, pH = 7.4, 37°C before exposing the myocytes to simulated ischemia. Simulated ischemia was induced by exposing the myocytes to 90 min of hypoxia and glucose deprivation in a hypoxic incubator (NuAire), where O_2 was replaced by N_2 as previously described (11, 12). Determination of myocyte injury was made at the end of the simulated ischemia, at which time myocytes were removed from the hypoxic incubator and reexposed to room air (normal % O_2). Aliquots of the media were then obtained for creatine kinase activity measurement, followed by quantitation of the number of viable cells. In parallel, ATP content was quantitated by rapid freezing in liquid nitrogen, followed by perchloric acid treatment, neutralization, centrifugation, and bioluminescent measurement. The light emitted after firefly luciferase-catalyzed oxidation of D-luciferin by ATP was determined in a luminometer (15, 16). The ATP level was derived from an ATP calibration curve constructed by quantitating the relative light intensity versus known amounts of ATP. Measurement of the basal level of cell injury was made after parallel incubation of control cells under normal % (room air) O_2. The extent of ischemia-induced myocyte injury was quantitated as decreased ATP content, increased number of cells killed, and amount of creatine kinase (CK)[2] released. The amount of CK was measured as enzyme activity (unit/mg); increases in CK activity above the control level were 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 control group multiplied by 100%.

Preconditioning of the cardiac myocyte was achieved as previously described (14). Briefly, preconditioning was induced by exposing the myocytes to 5 min of simulated ischemia, termed preconditioning ischemia, which was followed by an intervening 10 min normoxia and then by 90 min of simulated ischemia. Myocytes not subjected to preconditioning were exposed to 90 min ischemia only (nonpreconditioned cells). For both preconditioned and nonpreconditioned cells, determination of cell injury was made at the end of the 90 min ischemic period.

Gene transfer into cardiac myocytes

Cardiac ventricular myocytes were transfected with the various recombinant pcDNA3 vectors using a newly modified calcium phosphate precipitates method (17). Human cDNAs encoding the adenosine A_1 receptor (hum A_1AR) and the adenosine A_3 receptor (hum A_3AR) (18, 19) were subcloned into the eucaryotic expression vector pcDNA3, termed pcDNA3/hum A_1AR and pcDNA3/hum A_3AR. Cardiac myocytes were maintained in culture for 24 h prior to being exposed to the calcium phosphate/DNA precipitates for 6 h at 37°C. Media were replaced with fresh growth media after two washes, and the myocytes were cultured for an additional 48 h. The efficiency of gene transfer was correlated with the formation of coarse precipitates produced by increasing the calcium phosphate concentration in the transfection cocktail.

[2]Abbreviations: ANOVA, analysis of variance; CK, creatine kinase; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CCPA, 2-chloro-N^6-cyclopentyladenosine; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide; MRS 1191, 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydro-pyridine-3,5-dicarboxylate; Ado, adenosine.

Expression of foreign DNAs as functional proteins

Expression of the foreign DNAs as functional proteins was assayed 48 h after the transfection procedure. Lac-Z positive myocytes were identified by X-gal (5-bromo-4-chloro-indolyl-β-galactopyranoside) determination and were quantitated by counting as previously described (17). The expression of human adenosine A_1 and A_3 receptor cDNAs as functional proteins was determined by the ability of transfected human adenosine A_1 or A_3 receptor to mediate inhibition of iso-proterenol-stimulated adenylyl cyclase. An enhanced A_1 agonist-mediated inhibition of cyclic AMP accumulation in myocytes transfected with the A_1 receptor cDNA indicated its expression as a functional A_1 receptor. Similarly, an increased A_3 agonist-mediated inhibition of cyclic AMP accumulation in A_3 receptor DNA-transfected myocytes served to indicate its expression as a functional receptor. Adenosine receptor-mediated inhibition of isoproterenol-stimulated cyclic AMP accumulation was determined as previously described (14, 20). Finally, whether transfection of the myocytes with A_1 or A_3 receptor cDNA conferred a cardioprotective effect that is sensitive to blockade by A_1 or A_3 receptor-selective antagonists, respectively, was also determined. This served as an additional indication of whether the foreign DNAs are expressed into functional proteins.

Materials

The adenosine analogs 2-chloro-N^6-cyclopentyladenosine (CCPA), 8-sulfophenyltheophylline (8-SPT), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were from Research Biochemicals International (Natick, Mass.). N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA) and 3-ethyl5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydro-pyridine-3,5-dicarboxylate (MRS 1191) were synthesized as described (21, 22). Adenosine was obtained from Sigma Chemical Co. (St. Louis, Mo.). The vector pcDNA3 was obtained from Invitrogen (Carlsbad, Calif.). Embryonated chick eggs were from Spafas, Inc. (Storrs, Conn.).

RESULTS

Effects of adenosine and adenosine agonists on the level of ATP, the amount of CK released, and the percentage of myocytes killed

Simulated ischemia, induced by hypoxia and glucose deprivation, caused a reduction in ATP content and an increased release of creatine kinase into the media (Fig. 1) as well as a large percentage of myocytes killed (31±3%, n=6, ±SE). ATP content is inversely related to the extent of cardiac myocyte injury. The presence of exogenous adenosine (10 μM) or, more specifically, of the adenosine A_1 receptor agonist CCPA (10 nM) or the A_3 receptor agonist IB-MECA (30 nM), attenuated the extent of myocyte injury. All three agonists, present at maximally cardioprotective concentrations, preserved cellular ATP content, decreased the release of creatine kinase (Fig. 1), and reduced the number of myocytes killed (data not shown). These data showed that the level of ATP content is inversely correlated with the level of creatine kinase or the percentage of myocytes killed and suggest that preservation of ATP content is a useful index of myocyte protection. The cardioprotective effect of CCPA was completely blocked by 1 μM of DPCPX (13) whereas the protective effect of IB-MECA was abolished by 1 μM of the A_3-selective antagonist MRS1191 (data not shown). The

presence of 100 μM 8-SPT during the 90 min ischemia, in the absence of A_1 or A_3 receptor agonist, had no effect on the extent of myocyte injury incurred during the prolonged ischemia (% cells killed and amount of CK released without 8-SPT=35.8±2.1% and 27.22±2.3. unit/mg, with 8-SPT=30.4±4.2% and 23.2±2 unit/mg; n=11, ±SE, P>0.1).

Effect of expressing the human adenosine A_3 receptor on the resistance to injury-producing ischemia

Whether an increased expression of the adenosine A_3 receptor in cardiac myocytes can protect them against ischemia-induced injury was investigated next. Using a newly modified calcium precipitates-based method of gene transfer (14), the cultured cardiac myocytes can be efficiently transfected with over 40% of the myocytes (43±4%, nn=four cultures) taking up and expressing the Lac-Z DNA (Fig. 2). Whether the human adenosine A_3 receptor cDNA, upon transduction in the cardiac myocytes, can be expressed as a functional receptor was determined. This was accomplished by quantitating the extent of IB-MECA-mediated inhibition of cyclic AMP accumulation in myocytes transfected with the A_3 receptor cDNA. Cardiac myocytes transfected with the A_3 receptor cDNA had an enhanced IB-MECA-mediated inhibition of adenylyl cyclase activity. The percent of inhibition of isoproterenol-stimulated cyclic AMP accumulation by IB-MECA in pcDNA3/hum A_3AR-transfected myocytes was 21 ±3% (n=4) vs. 14 ±4% (n=4) in pcDNA$_3$-transfected myocytes (P<0.05, t test).

During the 90 min ischemia, myocytes transfected with the human adenosine A_3 receptor cDNA had greater ATP content, fewer cells killed, and less CK released than did untransfected myocytes or myocytes transfected with pcDNA3 [one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison test, P<0.01] (Fig. 3 and Fig. 4). Thus, transfection with pcDNA3/hum A_3AR resulted in functional expression of the human adenosine A_3 receptor cDNA. An increased expression of the A_3 receptor rendered the myocytes resistant to the deleterious effect of ischemia.

Enhanced preconditioning effect in A_3 receptor DNA-transfected myocytes

The myocyte model of simulated ischemia was further broadened to a model of ischemic preconditioning (13) and the effect of increased adenosine A_3 receptor expression on preconditioning-induced protection was determined. Five minutes of exposure to the preconditioning ischemia resulted in fewer cells killed and less CK released in cultures transfected with the A_3 receptor cDNA as compared to cultures transfected with pcDNA3 (one-way ANOVA and Student-Newman-Keuls multiple comparison test, P<0.01) (Fig. 4*A, B*). Thus, enhanced expression of the A_3 receptor also led to an enhanced cardioprotective effect by the preconditioning ischemia.

Effects of adenosine A_1 receptor overexpression on the myocyte resistance to ischemia

Because activation of the A_1 receptor can also induce cardioprotection, parallel experiments to examine the effects of overexpressing the human adenosine A_1 receptor were also carried out. Myocytes transfected with pcDNA3/hum A_1AR had enhanced A_1 agonist-mediated inhibition of cyclic AMP accumulation. The percent of inhibition of isoproterenol-stimulated cyclic AMP accumulation induced by CCPA in pcDNA3/hum A_1AR-transfected

myocyte cultures was 22 ±4% (n=4, ±SE) (P<0.05, t test vs. percentage of inhibition in pcDNA3-transfected myocytes). These data, consistent with the finding that transfection with the A_1 receptor cDNA resulted in an enhanced inhibition of isoproterenol-stimulated myocyte contractility by the A_1 receptor agonist (17), demonstrate the expression of exogenous A_1 receptor cDNA as a functional protein. Myocyte cultures transfected with the human adenosine A_1 receptor cDNA had greater ATP content, fewer cells killed, and less CK released than cultures transfected with pcDNA3 (one-way ANOVA and Student-Newman-Keuls multiple comparison test, P<0.01) (Fig. 3 and Fig. 4). Preconditioning ischemia also produced fewer cells killed and less CK released in myocytes transfected with pcDNA3/hum A_1 AR compared with myocytes transfected with pcDNA3 (one-way ANOVA and Student-Newman-Keuls multiple comparison test, P<0.01) (Fig. 4). Thus, overexpressing the A_1 receptor resulted not only in increased protection against injury-producing ischemia, but also an enhanced preconditioning effect.

Although increased expression of the A_1 or A_3 receptor protected against myocyte injury during the 90 min ischemia, the extent of protection was less than that produced by maximally cardioprotective concentration of the A_1 or A_3 receptor agonist. Thus, the ischemia-induced killing of myocytes and release of CK were significantly greater in A_1 receptor-over-expressing myocytes than in A_1 agonist-treated myocytes (P<0.05, t; test). Similarly, after the ischemia exposure, the percentage of cells killed and the amount of CK released were also significantly higher in A_3 receptor-expressing myocytes than in A_3 agonist-treated myocytes (P<0.05, t; test). In fact, exposure of A_1 receptor-overexpressing myocytes to 10 nM CCPA during the 90 min ischemia caused a further reduction in the amount of CK released and the number of cardiac cells killed as compared to the A_1 receptor cDNA-transfected myocytes that had not been exposed to CCPA (Fig. 5). Similarly, incubation of pcDNA3/hum A_3AR-transfected myocytes with IB-MECA (30 nM) during the 90 min ischemia resulted in a lower level of CK released and fewer cardiac cells killed than the A_3 receptor cDNA-transfected myocytes that were not exposed to IB-MECA (Fig. 5).

Receptor-selective antagonists abolished the cardioprotection achieved by increased receptor expression

The next question concerns whether the protection against ischemia-induced injury in pcDNA3/hum A_1AR- or pcDNA3/hum A_3AR-transfected myocytes is mediated by the A_1 or A_3 receptor, respectively. To test this notion, the adenosine A_1 receptor-selective antagonist DPCPX was included in the medium during exposure of pcDNA3/hum A_1AR-transfected myocytes to the prolonged ischemia. If the cardioprotective effect conferred by transfection is due to expression of the exogenous A_1 receptor cDNA, the protection should be abolished by the A_1 selective antagonist DPCPX. Figure 6 demonstrates that this is indeed the case. On the other hand, the presence of A_3 receptor-selective antagonist MRS1191 (1 μM) during the 90 min ischemia did not attenuate the cardioprotection achieved by the enhanced A_1 receptor expression (ATP level without MRS1191 n=42 ±2 nmol/mg; ATP level plus MRS1191 n=41 ±3 nmol/mg, ±SE, n=4. P>0.5, t test)

Whether the cardioprotection achieved by an increased expression of the A_3 receptor can be abolished by MRS1191 but remain unaffected by DPCPX was determined. The A_3 receptor-

selective antagonist MRS1191 abolished the cardioprotective effect due to transfection with pcDNA3/hum A_3AR (Fig. 6). However, the presence of DPCPX (1 μM) during the 90 min ischemia did not significantly affect the extent of protection against ischemia-induced injury in myocytes expressing the human A_3 receptor (ATP level without DPCPX n=40.3±2.0 nmol/mg; ATP level plus DPCPX n=39.5±1.8 nmol/mg; P>0.5, t test). Taken together, these data indicate that the resistance to ischemia is mediated by the exogenous adenosine A_1 or A_3 receptors after transfection and expression of their DNAs in the cardiac myocyte.

DISCUSSION

Adenosine is released in large amounts during myocardial ischemia and functions as a potent cardioprotective agent capable of reducing the extent of myocardial injury (1–6). Adenosine can achieve two types of cardioprotection. The first is its role in triggering and mediating the protective effect of ischemic pre-conditioning. The second concerns its ability to protect against myocyte injury when it is present during an infarct-producing ischemia. Two approaches can be used to achieve these cardioprotective effects. One involves the administration of a receptor-selective agonist to activate the receptor. The other approach is to increase the receptor number in the myocardium so that its sensitivity to the endogenous adenosine can be enhanced.

In the present study, a previously established cardiac myocyte model of ischemia-induced injury (13, 14) was used to test the hypothesis that genetic manipulation of the cardiac myocyte, achieved by gene transfer and overexpression of the human A_3 receptor cDNA, renders the myocytes more resistant to the deleterious effect of ischemia. Prolonged hypoxia with glucose deprivation, causing myocyte injury and adenosine release, was used to simulate ischemia in cultured chick embryo ventricular myocytes. The model system developed provided a unique opportunity to test this hypothesis.

The most significant, novel finding is that during ischemia, cardiac myocytes overexpressing the human A_3 receptor showed significantly higher ATP content, fewer cells killed, and less CK release than either control or mock-transfected myocytes. The myocyte model was broadened to a model of ischemic preconditioning (14) and the effect of increased receptor expression on preconditioning-induced protection was determined. After exposure to preconditioning ischemia, cardiac myocytes transfected with the A_3 receptor DNA had fewer cells killed and less CK released than myocytes transfected with the vector during the subsequent 90 min ischemia. Thus, increasing the level of myocyte adenosine A_3 receptor conferred an enhanced cardioprotective effect by the preconditioning ischemia.

Enhanced expression of the human adenosine A_1 receptor also led to increased protection against ischemia-induced myocyte injury. This protection was consistent with that obtained in transgenic mouse overexpressing the rat adenosine A_1 receptor in that the transgenic hearts were more resistant to ischemia than the control hearts (11). However, the present data also indicated that overexpressing the A_1 receptor can also confer an increased cardioprotective effect by the preconditioning ischemia. Although the relative advantage of overexpressing the A_3 vs. the A_1 receptor in achieving cardioprotection is still under

investigation, the present data suggest that both human adenosine A_1 and A_3 receptors are new therapeutic targets in treating myocardial ischemia.

Two lines of evidence further confirm the expression of the human A_1 and A_3 receptor cDNAs as functional proteins. First, myocytes transfected with A_1 or A_3 receptor cDNA enhanced A_1 or A_3 agonist-mediated inhibition of the cyclic AMP level, respectively. Second, the cardioprotective effect in A_1 or A_3 receptor DNA-transfected myocytes was abolished by the presence of A_1 or A_3 receptor antagonist, respectively, during the prolonged ischemia. The data suggest that the cardioprotective effect conferred by transfection is due to expression of the exogenous receptor c-DNAs as functional receptors.

Although increased expression of adenosine A_1 or A_3 receptors protected the cardiac myocytes against injury produced by the sustained ischemia, the extent of cardioprotection is less than that produced by the maximally effective concentration of CCPA or IB-MECA. The reason for this difference is not clear but is likely due to the fact that only a fraction of the myocytes were transfected. Another possibility is that although the levels of A_1 and A_3 receptors are increased compared to vector-transfected myocytes, the increase is not enough to achieve maximal sensitivity to the endogenous adenosine released during the ischemia. These notions are also supported by the finding that the extent of cardioprotection can be increased further by exposing A_1 or A_3 receptor cDNA-transfected myocytes to CCPA or IB-MECA, respectively. Overall, the present data indicate that increasing the receptor level enhances the myocyte sensitivity to the endogenous adenosine, which in turn causes all of the cardioprotective effects found for exogenously administered adenosine agonists. The present study provides the first proof of the novel concept that overexpression of the adenosine A_3 receptor in cardiac myocytes can render them more ischemia resistant. The data suggest that overexpression of the human A_3 receptor in the myocardium can be an important cardioprotective therapeutic approach.

Acknowledgments

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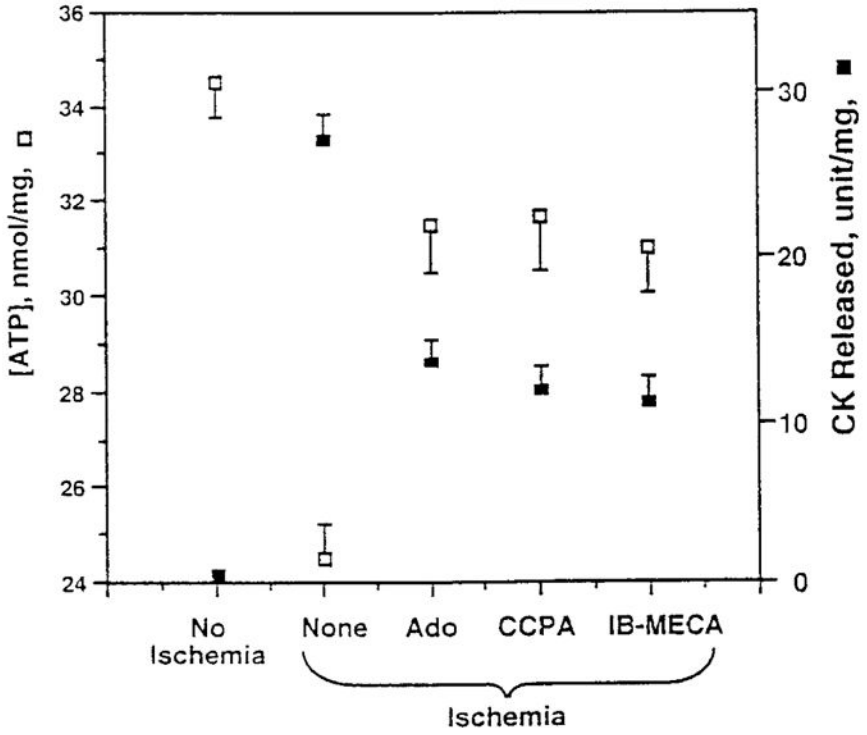

Figure 1.

Activation of the adenosine A_1 and A_3 receptors can preserve myocyte ATP level and decrease injury during prolonged simulated ischemia. Cardiac ventricular myocytes were cultured and exposed to simulated ischemia as described in Materials and Methods. Adenosine (10 μM), the A_1 receptor-selective agonist CCPA (10 nM), or the A_3 receptor-selective agonist IB-MECA (30 nM) was added to media just before the prolonged simulated ischemia. The level of ATP and amount of creatine kinase released were determined at the end of the 90 min ischemia. Data represented means + $_{SE}$ of five experiments. The presence of adenosine, CCPA, or IB-MECA during the 90 min ischemia preserved the level of ATP and decreased the amount of CK released compared to myocytes treated with no adenosine or agonist (one-way ANOVA and Student-Newman-Keuls multiple comparison test, $P<0.001$).

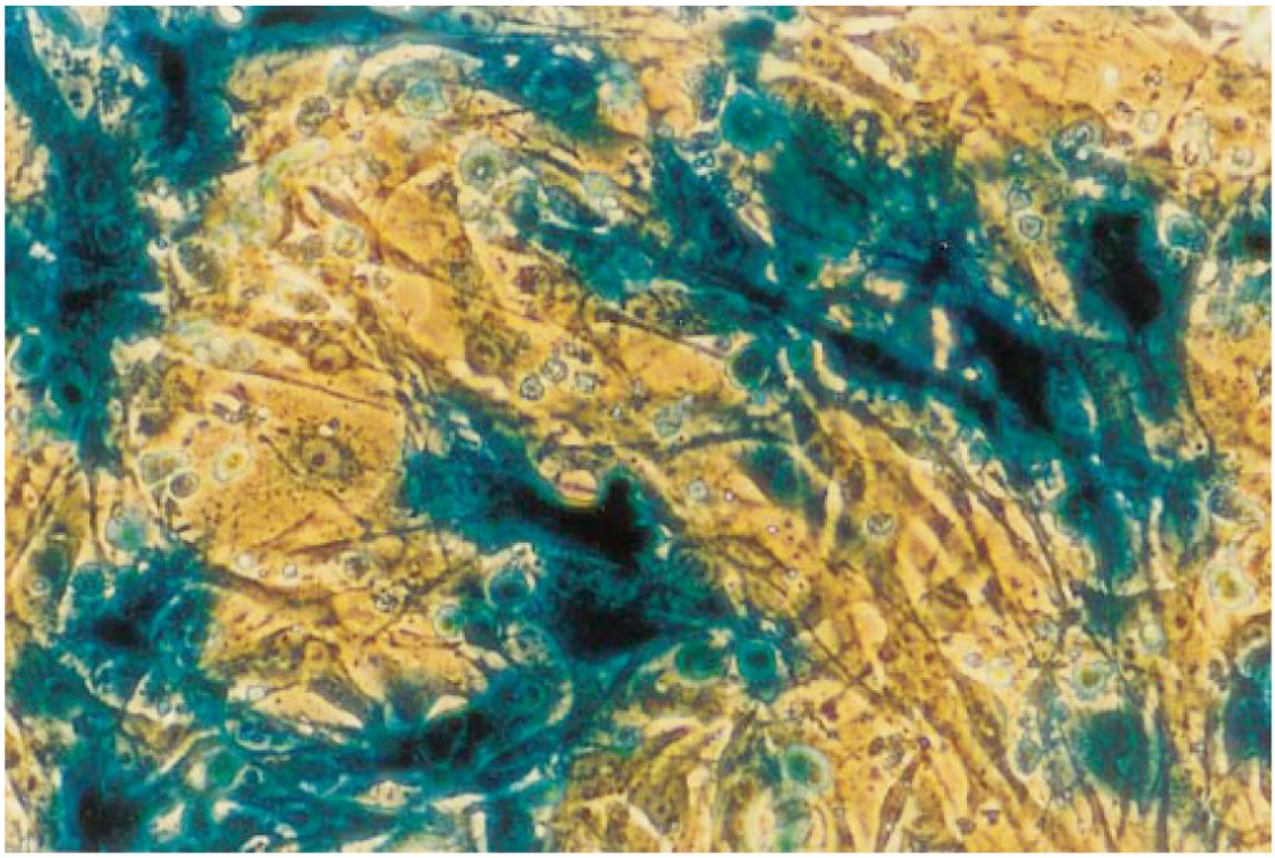

Figure 2.
Efficient gene transfer into the cardiac myocyte. Cardiac ventricular myocytes were cultured and transfected 24 h later. The Lac Z-positive myocytes were identified and quantitated 48 h after the transfection. The data were typical of three other experiments performed on three cultures.

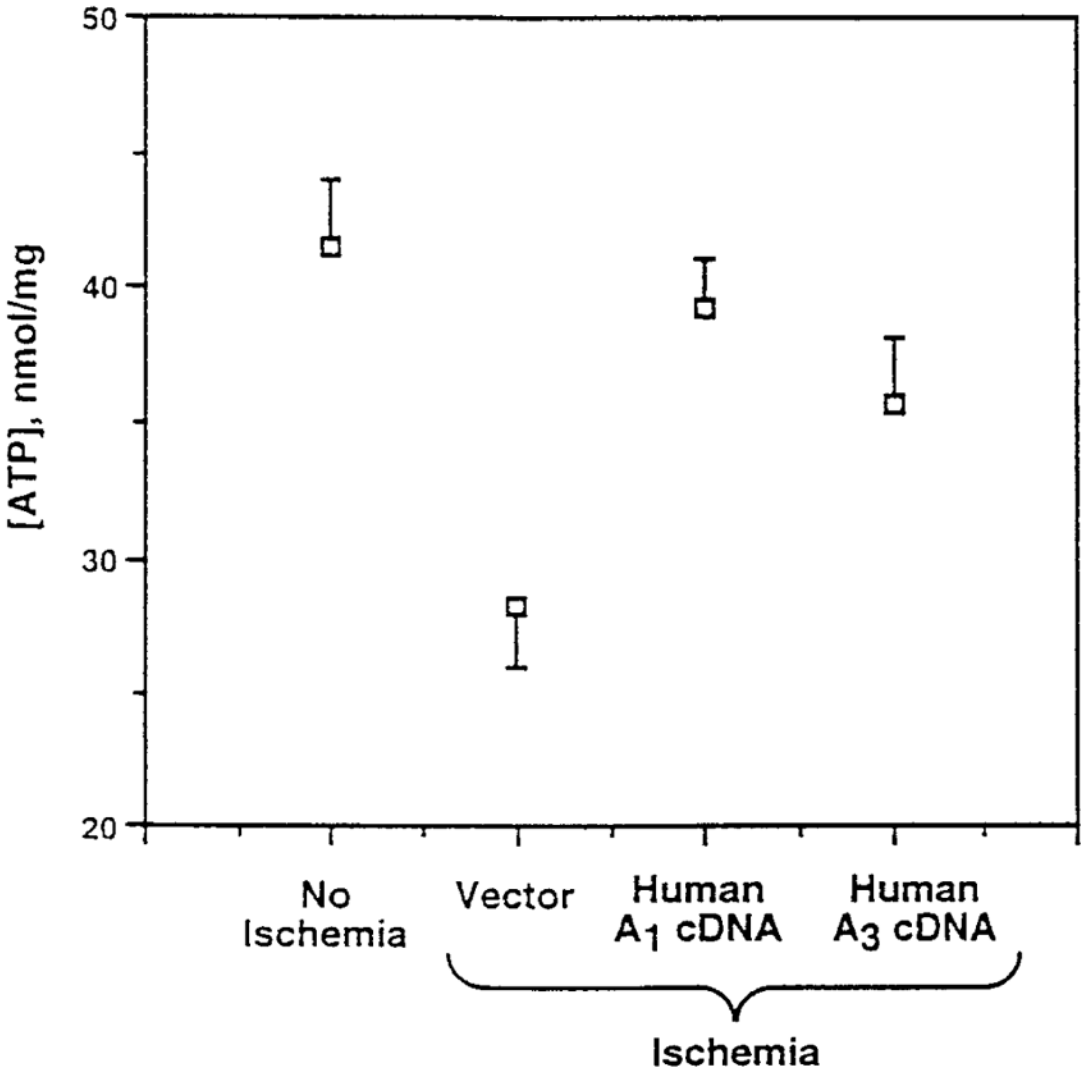

Figure 3.

Transfection with human adenosine A_1 or A_3 receptor cDNAs preserved myocyte ATP content during ischemic exposure. Cardiac ventricular myocytes were cultured and transfected with pcDNA3, pcDNA3/hum A_1AR or pcDNA3/hum A_3AR. Forty eight hours after the transfection, myocytes were exposed to the simulated ischemia protocol as described in Materials and Methods. The level of ATP was determined at the end of the ischemia. Data were the means and standard errors of triplicate determinations and were representative of five experiments. Myocytes transfected with the A_1 receptor or A_3 receptor cDNA had significantly higher ATP level than myocytes transfected with the vector (one-way ANOVA analysis, followed by Student-Newman-Keuls multiple comparison test, $P<0.01$).

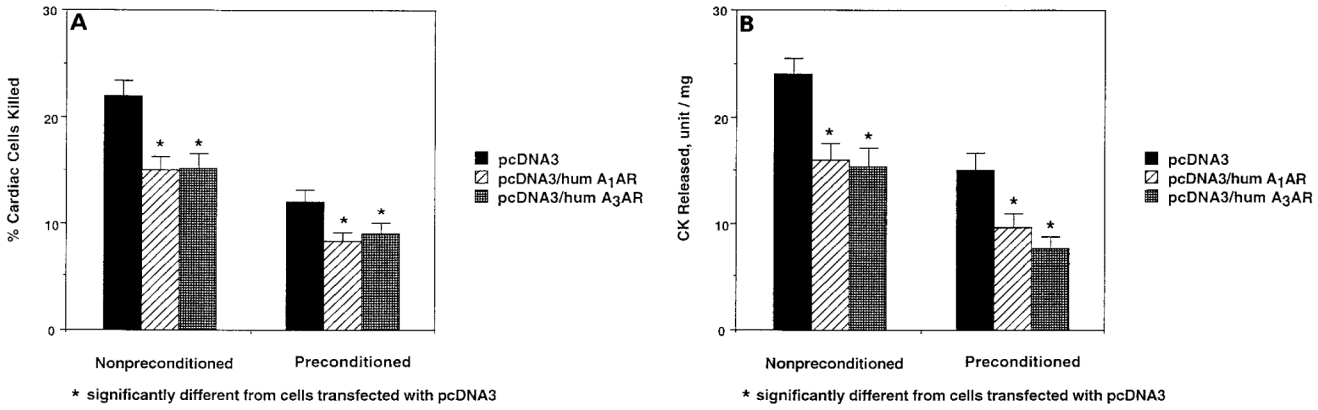

Figure 4.

Effects of overexpressing the adenosine A_1 or A_3 receptor on the cardioprotective effect of preconditioning ischemia. Ventricular myocytes were transfected with pcDNA3, pcDNA3/hum A_1AR (pcDNA3/hA_1AR), or pcDNA3/hum A_3AR (pcDNA3/hA_3AR) as described in Materials and Methods. Transfected myocytes were then exposed to 90 min simulated ischemia and represented nonpreconditioned cells. In parallel experiments, the transfected myocytes were preconditioned by a 5 min exposure to simulated ischemia, which was followed by 10 min of normoxia and then by 90 min of simulated ischemia. The amount of CK released and the percentage of cells killed were determined at the end of 90 min ischemia in both preconditioned and nonpreconditioned myocytes. Brief exposure to preconditioning ischemia caused protection against injury produced by the subsequent 90 min ischemia in myocytes transfected with either pcDNA3, pcDNA3/hA_1AR, or pcDNA3/hA_3AR. For all transfected myocytes, the percentage of cells killed and the amount of CK released were significantly less in preconditioned than in nonpreconditioned cells (one-way ANOVA analysis, followed by Student-Newman-Keuls multiple comparison test, $P<0.01$). Data represented means $\pm_{SE}$ of seven experiments. *Significantly different from cells transfected with pcDNA3 (one-way ANOVA and Student-Newman-Keuls multiple comparison test, $P<0.01$).

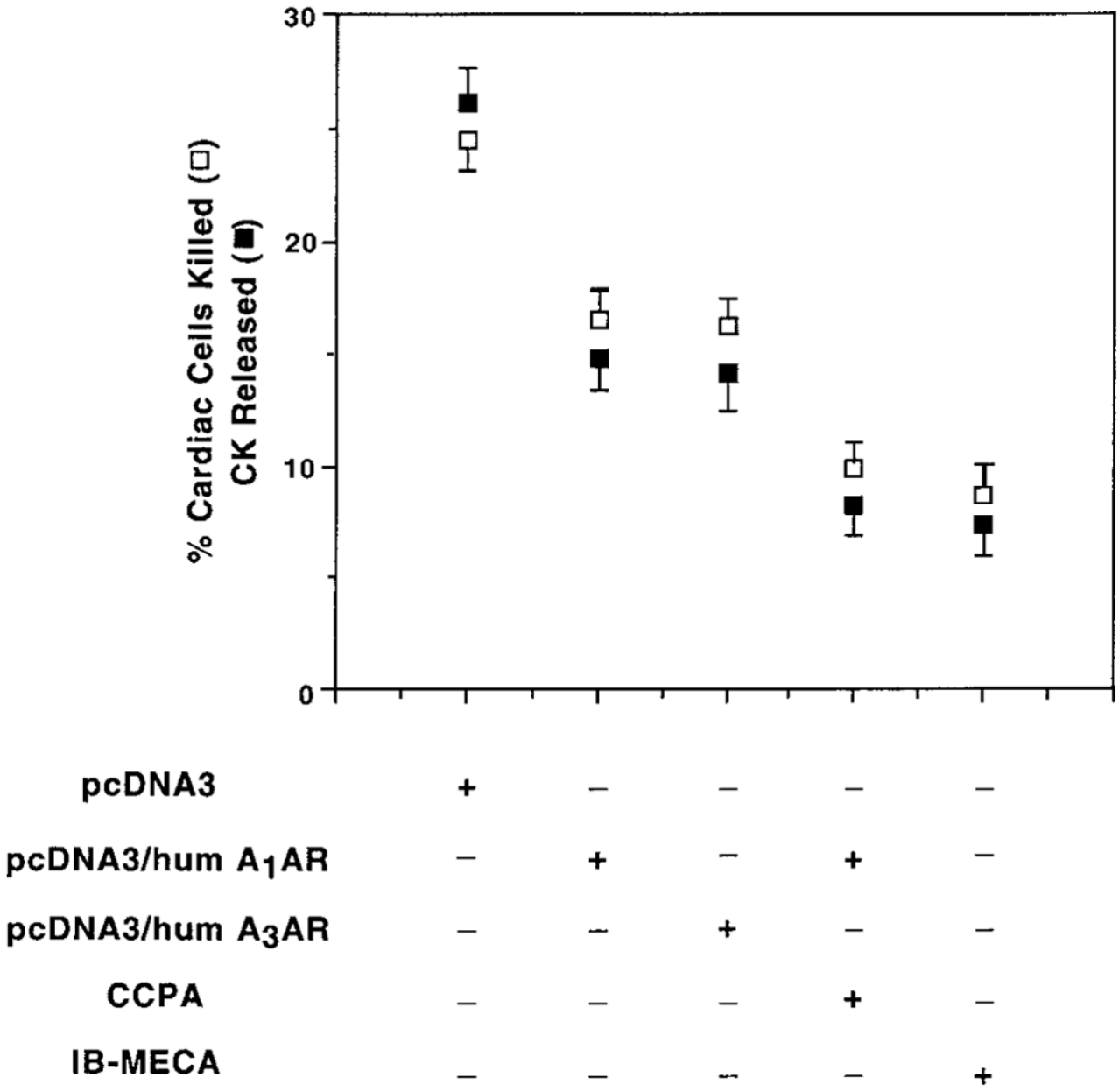

Figure 5.

CCPA and IB-MECA enhanced the cardioprotection achieved by increased expression of the adenosine A_1 and A_3 receptor, respectively. Ventricular myocytes were transfected with pcDNA3, pcDNA3/hum A_1AR (pcDNA3/hA_1AR), or pcDNA3/hum A_3AR (pcDNA3/hA_3AR) and exposed to 90 min ischemia as described in Materials and Methods. To test whether the addition of exogenous agonist would further enhance the cardioprotection achieved by increased receptor expression, myocytes transfected with pcDNA3/hum A_1AR and pcDNA3/hum A_3AR were then exposed to 90 min simulated ischemia in the presence of CCPA (10 nM) or IB-MECA (30 nM), respectively. The presence of CCPA caused fewer cells killed and less CK released compared to cells not incubated with CCPA (one-way

ANOVA analysis, followed by Student-Newman-Keuls multiple comparison test, $P<0.001$). Similarly, exposure of pcDNA3/hum A_3AR-transfected cells to IB-MECA during the sustained ischemia also resulted in fewer cells killed and less amount of CK released (one-way ANOVA and Student-Newman-Keuls multiple comparison test, $P<0.001$).

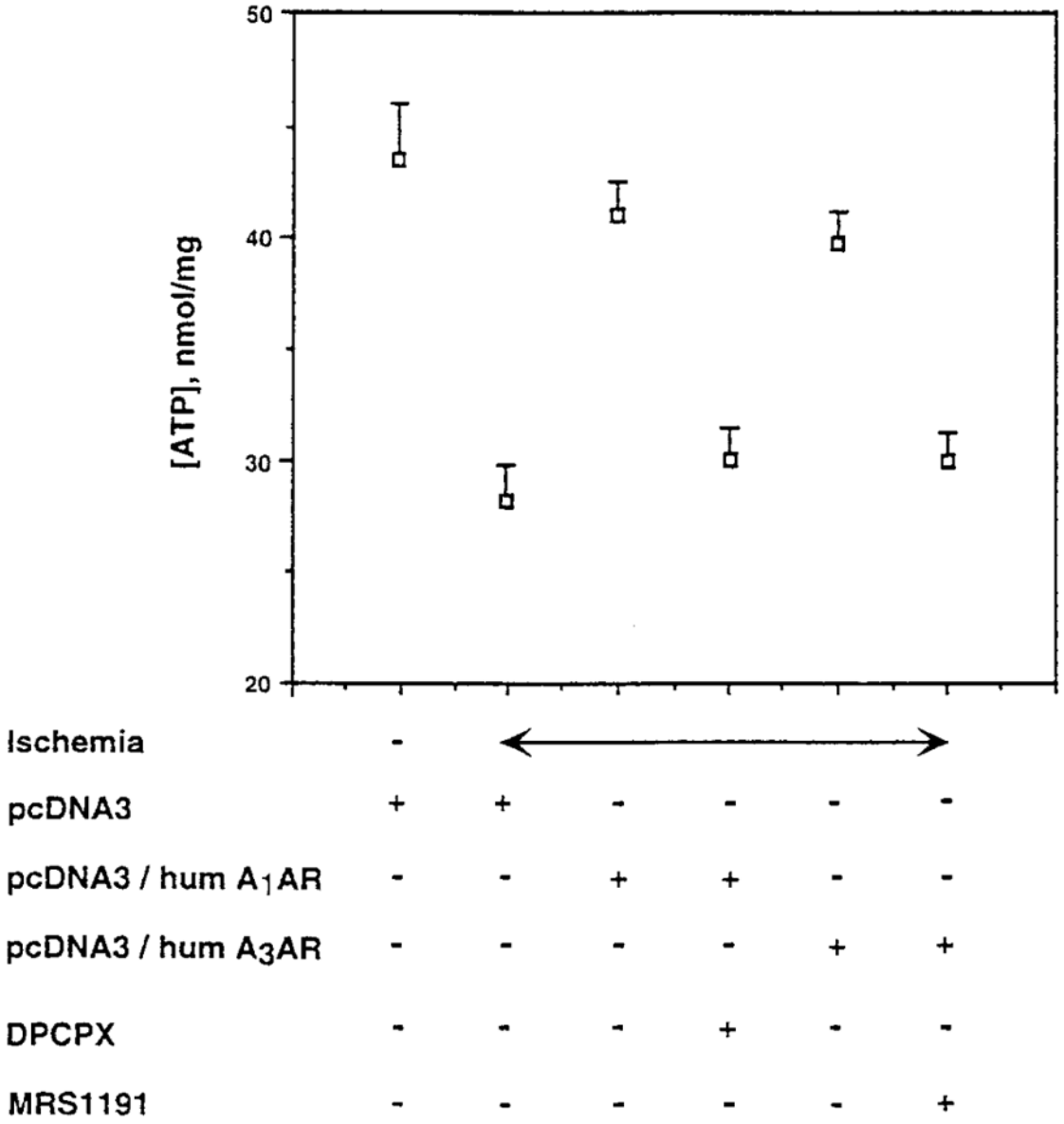

Figure 6.

Effects of DPCPX and MRS1191 on the cardioprotection in pcDNA3/hum A_1AR- and pcDNA3/hum A_3AR-transfected cardiac myocytes, respectively. Cardiac ventricular myocytes were cultured and transfected with pcDNA3/hum A_1AR and pcDNA3/hum A_3AR as described in legend to Fig. 3. After 48 h in culture, myocytes transfected with pcDNA3/hum A_1AR or pcDNA3/hum A_3AR were exposed to ischemia in the presence of DPCPX (1 μM) or MRS1191 (1 M), respectively. The level of ATP was determined at the end of ischemia. Data were the means and standard errors of triplicate determinations and were typical of three other experiments. In pcDNA3/hum A_1AR-transfected myocytes, the ATP content was significantly lower in the presence than in the absence of DPCPX (1 μM) (one-way ANOVA and Student-Newman-Keuls multiple comparison test, $P<0.001$). Similarly, in pcDNA3/hum A_3AR-transfected myocytes, the ATP level was significantly

lower in the presence than in the absence of MRS1191 (1 μM) (one-way ANOVA and Student-Newman-Keuls multiple comparison test, $P<0.01$). The presence of DPCPX or MRS1191 reduced the ATP level to that observed in pcDNA3-transfected myocytes.