Direct Preconditioning of Cultured Chick Ventricular Myocytes

Novel Functions of Cardiac Adenosine A_{2a} and A_3 Receptors

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Abstract

Preconditioning with brief ischemia before a sustained period of ischemia reduces infarct size in the perfused heart. A cultured chick ventricular myocyte model was developed to investigate the role of adenosine receptor subtypes in cardiac preconditioning. Brief hypoxic exposure, termed preconditioning hypoxia, prior to prolonged hypoxia, protected myocytes against injury induced by the prolonged hypoxia. Activation of the adenosine A_1 receptor with CCPA or the A_3 receptor with Cl-IB-MECA can replace preconditioning hypoxia and simulate preconditioning, with a maximal effect at 100 nM. While activation of the A_{2a} receptor by 1 μM CGS21680 could not mimic preconditioning, its stimulation during preconditioning hypoxia, however, attenuated the protection against hypoxia-induced injury. Blockade of A_{2a} receptors with the selective antagonist CSC (1 μM) during preconditioning hypoxia enhanced the protective effect of preconditioning. Nifedipine, which blocked the A_{2a} receptor-mediated calcium entry, abolished the A_{2a} agonist-induced attenuation of preconditioning. Isoproterenol, forskolin, and BayK 8644, which stimulated calcium entry, also attenuated preconditioning. Nifedipine blocked the increase in calcium uptake by these agents as well as their attenuating effect on preconditioning. The present study provides the first evidence that the adenosine A_3 receptor is present on ventricular myocytes and can mediate simulation of preconditioning. The data demonstrate, for the first time, that activation of the A_{2a} receptor antagonizes the preconditioning effect of adenosine, with increased calcium entry during the preconditioning stimuli as a novel mechanism. (*J. Clin. Invest.* 1996. 98:1773–1779.) Key words: myocardium • adenosine • receptor • purinergic • ischemia

Introduction

Preconditioning has been demonstrated in isolated perfused hearts of a number of mammalian species including dog, guinea pig, pig, rabbit, and rat (1–6, 10–13). Indirect evidence for such protective preconditioning also exists in humans (5, 6, 14). Adenosine is released in large amounts during myocardial ischemia and has been demonstrated to play a major role in mediating preconditioning and other cardioprotective effects in most animal species, including humans (1–14). Previous studies (15–17) of adult human and rabbit ventricular myocytes and cultured neonatal rat cardiac myocytes provided important insight by indicating that the cardioprotective mechanism of preconditioning is exerted, at least in part, at the level of and by the cardiac myocytes in the intact heart. Although a non-A_1 receptor, possibly the A_3 subtype, may be involved in mediating preconditioning, very little is known regarding the role of different adenosine receptor subtypes in mediating or modulating preconditioning of the cardiac myocytes.

Ventricular myocytes cultured from chick embryos retain many of the properties of the intact heart and have served as a useful model for a variety of experimental paradigms (18–24). Previous studies have demonstrated that activation of adenosine receptors in these cultured heart cells produces physiologic effects similar to those elicited by adenosine in the adult mammalian heart (25–28). The cultured ventricular myocytes contain predominantly (> 90%) myocytes (21; our unpublished data) and are largely devoid of neuronal, blood, or vascular cells, thus the confounding influence of changes in blood flow is avoided (18–28). The present study aims to develop the chick ventricular cell culture as a model to investigate the role of adenosine receptor subtypes in the preconditioning process and to study the mechanism(s) by which preconditioning of the ventricular myocytes can be modulated. To simulate preconditioning, ventricular myocytes were exposed to 5 min of hypoxia ($O_2 < 1\%$), reoxygenated in the presence of normal percent O_2 (room air) for 10 min, and then incubated in the presence of continuous hypoxia for 90 min ($O_2 < 1\%$). The development of a ventricular myocyte model for preconditioning and the use of a protocol identical to that employed in preconditioning of the isolated perfused heart facilitates cellular characterization of this phenomenon and enables quantitative determination of the extent of cardioprotection by preconditioning. The present study elucidates the role of A_1, A_{2a}, or A_3 adenosine receptors in mediating or modulating preconditioning and provides evidence that enhanced calcium entry during preconditioning hypoxia is a novel mechanism for attenuating the myocyte responsiveness to preconditioning stimuli.

Methods

Preparation and preconditioning of cultured ventricular cells. Ventricular cells were cultured from chick embryos 14 d in ovo, according to a previously described procedure (20, 27). Cells were plated at a density of 400,000 cells/ml and cultivated in a humidified 5% CO_2-95% air mixture at 37°C. All experiments were performed on day 3 in culture, at which time cells had grown to confluence and exhibited rhythmic

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spontaneous contraction. For preconditioning studies, the medium was changed to a Hepes-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 cells to the various conditions at 37°C. Control cells were maintained in the Hepes-buffered media under room air. Hypoxia was produced by placing the cells in a hypoxic incubator (NuAire) where O_2 was replaced by N_2. The percent O_2 was monitored by both an oxygen Fyrite Gas Analyzer (Bacharach, Pittsburgh, PA) and an oxygen analyzer (model OX630; Engineered Systems & Designs, Newark, DE). Preconditioning was induced by exposing the cells to 5 min of hypoxia, termed preconditioning hypoxia, prior to a second 90-min hypoxia. Cells not subjected to preconditioning were exposed to 90 min of hypoxia only (nonpreconditioned cells). The extracellular pH was similarly maintained at 7.4 by Hepes in both preconditioned and nonpreconditioned cells. Determination of basal level of cell injury was made after parallel incubation of control cells under normal percent O_2. For preconditioned and nonpreconditioned cells, determination of cell injury was made at the end of the 90-min hypoxic period.

Measurement of ^{45}Ca uptake and cyclic AMP level. Determination of ^{45}Ca uptake was made according to a modification of a previously described method (20). Cultures were incubated with L-(3, 4, 5-^3H, *N*)-leucine (152.2 Ci/mmol) for 24 h prior to ^{45}Ca uptake. [^3H] leucine incorporated into the cellular protein allowed normalization of ^{45}Ca content to mg cell protein. After incubation with ^{45}Ca-containing medium, cells were then washed free of ^{45}Ca and solubilized for 2 h with 1% sodium dodecyl sulfate and 10 mM sodium borate, and the levels of ^3H and ^{45}Ca were determined. For all data comparing the effect of different agents on ^{45}Ca uptake, one-way ANOVA analysis followed by group comparison with *t* test was carried out at each time point of the ^{45}Ca uptake. The cAMP level was determined according to a previously described radioimmunoassay (27).

Determination of cell injury. The extent of hypoxia-induced injury to the ventricular cell was quantitatively determined by the percentage of cells killed and by the amount of creatine kinase (CK)[1] released into the media. To quantitate the percent cells killed, cells were exposed to a trypsin-EDTA Hanks' balanced salt solution for 10 min for detachment after the various treatments, followed by centrifugation (300 *g* for 10 min) and resuspension in culture media for counting in a hemocytometer. Only live cells sedimented, and the cells counted represented those that survived (29). None of the sedimented cells subsequently counted included trypan blue. In cells not exposed to hypoxia, trypsin-EDTA treatment followed by reexposure to Ca^{2+}-containing culture media did not cause the appearance of trypan blue-stained cells or any significant increase in proteins or CK in the trypsin-EDTA media following the 300 *g*, 10-min sedimentation of the cells. There was no protein or CK in the culture media following a second 300 *g* centrifugation of resuspended cells previously treated with trypsin-EDTA. Thus, trypsin treatment, reexposure to Ca^{2+}-containing media or 300 *g* sedimentation did not cause any significant damage to the control, normoxia-exposed cells. In contrast, the trypsin-EDTA media from cells exposed to 90-min hypoxia contained substantial amounts of proteins (0.15±0.03 mg, $n = 9$) and CK activity (16±3 U/mg protein, $n = 8$). Such proteins and CK activity could arise from hypoxia-damaged cells which failed to sediment because of lighter cellular density from loss of soluble proteins or from hypoxia-exposed cells that were further damaged by the trypsin treatment. Because trypsin may cause further proteolysis of other proteins and CK, the amount of protein and CK retained by the damaged cells in the trypsin-EDTA media may not accurately reflect those associated with the damaged cells. In support of the notion that 90-min hypoxia caused significant cell injury and loss of membrane integrity, there was also marked release of LDH (hypoxia-exposed cells, 35.5±2.7, U/mg, $n = 8$, ±SE vs. control cells, 6.1±0.4 U/mg, $n = 8$) and proteins (hypoxia-exposed cells, 0.15±0.03 mg, $n = 8$ vs. control cells, 0.034±0.01 mg, $n = 8$) from the cells incubated under prolonged hypoxia. Thus, the cell viability assay separated out the hypoxia-damaged from the control normoxia-exposed cells. Parallel changes in percent cells killed and CK released (Fig. 1) further validated this assay for percent cells killed. The amount of CK was measured as enzyme activity (U/mg), and 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 number of cells in control group multiplied by 100%.

The pA_2 value for an antagonist at the A3 receptor was calculated by Schild plot in which log (X-1) was plotted versus -log [B] and the x-intercept is the pA_2 value. X = dose ratio which was the ratio of IC_{50} of the 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IB-MECA)-induced response in the presence of antagonist to the IC_{50} of the Cl-IB-MECA response in the absence of antagonist; B = antagonist concentration; pA_2 = -log K_d where K_d is the affinity constant for the antagonist. The pA_2 value was obtained from the line best fit by a slope value of −1.

Adenosine measurement and synthesis of adenosine receptor selective agonists. Adenosine concentrations in the buffer media were measured by an adenosine radioimmunoassay using antiserum specific to adenosine, which does not cross react with inosine or adenine nucleotides (30, 31). The recovery of adenosine standard, when added to the media containing the endogenous adenosine, was 96.4±7.2%, $n = 8$, ±SE. The electrolytes Na^+, K^+, and Cl^- were determined by ion-selective electrodes in a Boehringer Mannheim/Hita-

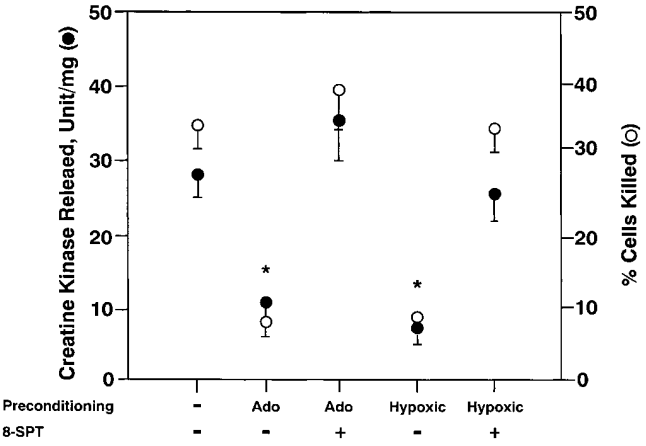

Figure 1. Preconditioning of ventricular cells by adenosine and hypoxia. Cells were preconditioned by a 5-min exposure to hypoxia or to 10 μM adenosine. In adenosine-treated cells, media were replaced by that lacking adenosine for 10 min prior to a 90-min hypoxic exposure. Effects of nonselective adenosine receptor antagonist 8-SPT (100 μM) on adenosine- or hypoxia-induced preconditioning, where 8-SPT was added during the 5-min exposure to adenosine or hypoxia, was also determined. The nonpreconditioned cells were exposed to 90 min of hypoxia only. Data were presented as percent cells killed (○) or as amount of CK released (●) and were mean±SE of six to eight experiments. * Difference from nonpreconditioned cells or from cells preconditioned in the presence of 8-SPT was statistically significant (one-way ANOVA analysis using *F* value, followed by group comparison employing unpaired *t* test where $P < 0.01$).

1. *Abbreviations used in this paper:* CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21680, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxiamidoadenosine; C1-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; CK, creatine kinase; CSC, 8-(3-chlorostyryl)caffeine; DPCPX, 8-cyclopentyl-1-1, 3-dipropylxanthine; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; R-PIA, N^6-R-phenyl-2-propyladenosine.

chi 747 analyzer using reagents supplied by Boehringer Mannheim (Indianapolis, IN) (32). 8-(3-chlorostyryl) caffeine (CSC), N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA) and Cl-IB-MECA were synthesized as described (33–35).

Results

Hypoxic preconditioning of the cultured ventricular cells. The cultured cells were preconditioned by a 5-min preconditioning hypoxia prior to a 90-min period of sustained hypoxia. The concentration of Na^+ (140 mM), K^+ (4.5 mM), Cl^- (145 mM), and the pH (7.44) of the buffer media did not change in control, preconditioned, or nonpreconditioned cells. The level of adenosine in culture media was determined next. In preconditioned cells, both 5-min and 90-min hypoxia produced significant increases in adenosine in the media (basal adenosine level: 21±2 nM, ±SE of triplicates; after 5-min hypoxia: 32±2.6 nM; after 90-min hypoxia: 70±5.5 nM; data were typical of nine experiments; ANOVA and paired *t* test $P < 0.01$). However, the extent of increase in adenosine produced by the 90-min hypoxia in preconditioned cells was less than that of the increase in nonpreconditioned cells (310±46%, $n = 10$, ±SE vs. 468±33%, $n = 10$; $P < 0.01$, *t* test), similar to the finding in preconditioning of the isolated perfused heart (36).

Exposure to 5-min hypoxia resulted in no significant increase in CK release or in any cell death (not shown). 90 min of hypoxic exposure resulted in a significant proportion of cells killed and a large increase in the release of CK into the media. Preconditioning hypoxia caused pronounced reductions in the percentage of cells killed (70.4±4%) and in the CK released (69±3%) (Fig. 1). There was minimal variability of proteins from one culture plate to another within the same culture (a typical culture has 0.9±0.03 mg protein per 60 mm dish, ±SD, $n = 20$ dishes). For all experiments, data on CK activity was normalized to total amount of protein content per 60 mm dish determined for each culture. The duration of the preconditioning effect was between 30 and 60 min after the preconditioning hypoxia but was lost at 60 min (data not shown).

Role of adenosine receptors in mediating preconditioning. To investigate the role of adenosine in mediating the preconditioning effect, the ventricular cells, instead of being exposed to the brief hypoxia, were incubated with adenosine for 5 min prior to the 90-min hypoxia. Adenosine did not cause any cell injury but elicited a significant attenuation of the injury produced by the subsequent 90-min hypoxia ($P < 0.01$ compared to nonpreconditioned cells, *t* test) (Fig. 1). The non-selective adenosine receptor antagonist 8-sulfophenyltheophylline (8-SPT, 100 μM), when added during exposure to adenosine or preconditioning hypoxia, completely abolished the preconditioning effect induced by adenosine or the preconditioning hypoxia, respectively (Fig. 1). The data indicate that adenosine receptor activation initiated the preconditioning. Continued activation of the adenosine receptor was required to sustain preconditioning because the presence of 8-SPT during the 90-min hypoxia was also able to completely abolish its protective effect. Such role of adenosine receptor is similar to that of the receptor in preconditioning of the intact heart (2, 3).

Adenosine A_{2a} receptor activation attenuates preconditioning. The adenosine receptor agonist R-PIA (Fig. 2), but not the A_{2a} receptor-selective agonist CGS21680 (not shown), could substitute for preconditioning hypoxia and induce preconditioning. However, compared to preconditioned cells, blocking

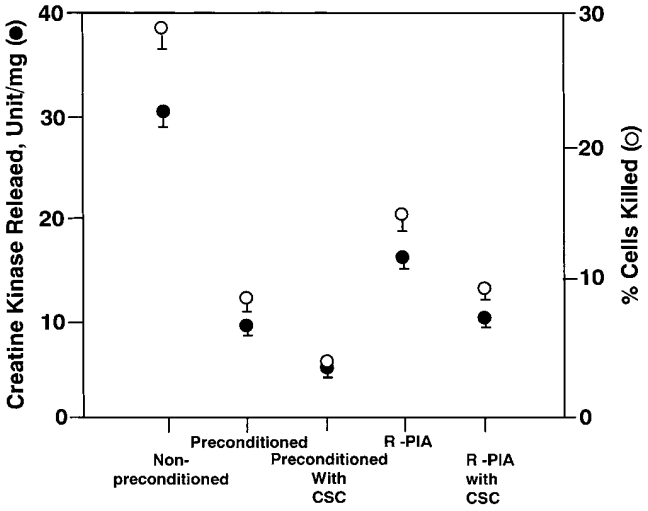

Figure 2. Effect of adenosine A_{2a} receptor activation on the protective effect of preconditioning hypoxia. Ventricular cells were preconditioned by a 5-min exposure to hypoxia in the presence or absence of the A_{2a} receptor-selective antagonist CSC (1 μM). Media, with or without the adenosine compounds, were obtained after the preconditioning hypoxia for CK measurement and were replaced with fresh media prior to 90-min hypoxia. The effect of CSC on R-PIA-induced preconditioning was also tested. Cells preconditioned by the 5-min treatment with 10 μM R-PIA in the presence or the absence of CSC were exposed to the 90-min hypoxia after R-PIA was removed by replacement with fresh media. Data were plotted as percent cells killed (○) or as amount of CK released (●). Data were the mean and standard errors of five experiments from five separate cultures.

of the adenosine A_{2a} receptor with its selective antagonist CSC (27, 32) during preconditioning hypoxia resulted in increased protection, that is, a further decrease in the percent cells killed and in the amount of CK released (ANOVA followed by group comparison using *t* test, $P < 0.01$) (Fig. 2). On the other hand, activation of the A_{2a} receptor by its agonist CGS21680 during preconditioning hypoxia attenuated the protection with an increase in the percent cells killed and in the amount of CK released (ANOVA and *t* test, $P < 0.01$) (Fig. 3). CSC enhanced the preconditioning effect induced by 10 μM R-PIA, which could activate not only the A_1 or A_3 receptors but also the A_{2a} receptor, with a further decrease in percent cells killed and in the amount of CK released compared to cells preconditioned by R-PIA alone (Fig. 2) ($P < 0.01$, *t* test). Prior exposure of the ventricular cells to CSC in the absence of preconditioning hypoxia had no effect on the extent of injury induced by the 90-min hypoxia (not shown). Such data further supported the notion that A_{2a} receptor activation can attenuate preconditioning.

Mechanism underlying the A_{2a} receptor-mediated attenuation of preconditioning. Activation of the adenosine A_{2a} receptor by CGS21680 (1 μM) led to nifedipine-sensitive increase in calcium entry. The levels of ^{45}Ca uptake after 90-s exposure to ^{45}Ca-containing media, in nmol/mg, were 4.2±0.1 for control, 5.8±0.2 for CGS21680 stimulated, and 4.4±0.1 for CGS21680 plus nifedipine (1 μM) stimulated cells (±SD, representative of five experiments). Thus, an increase in calcium entry during hypoxia- or R-PIA-induced preconditioning may be the mechanism by which A_{2a} receptor activation attenuated the protec-

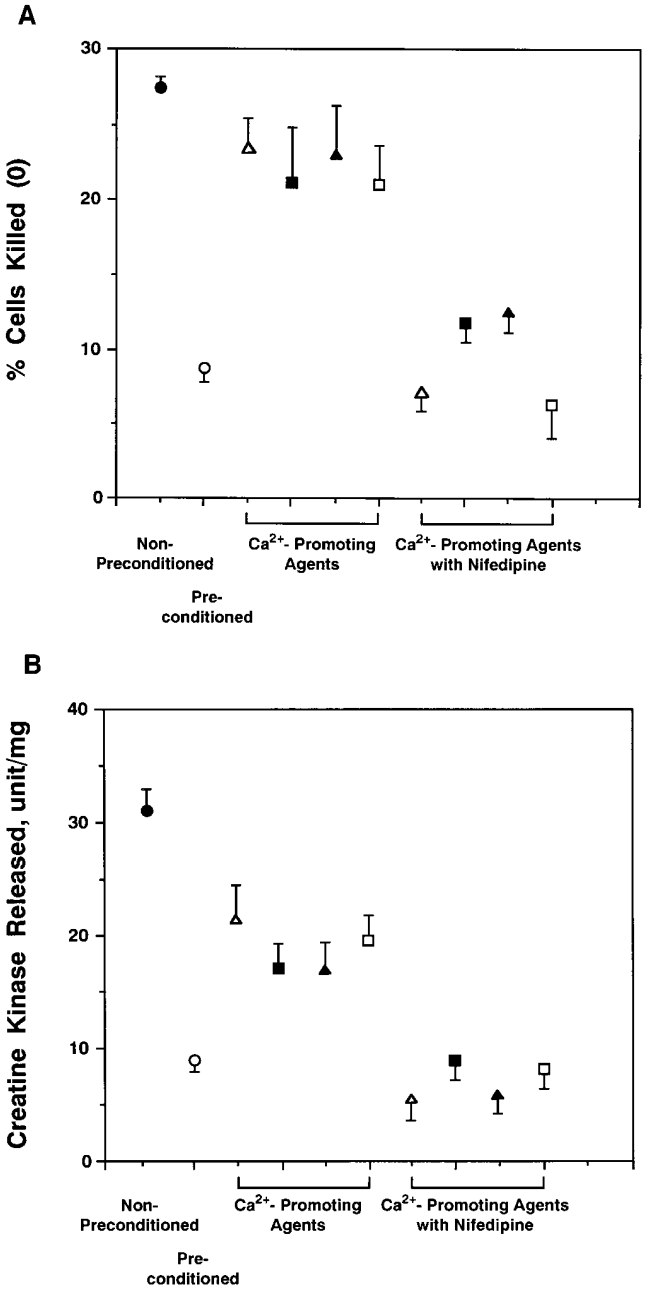

Figure 3. Nifedipine blocked the ability of CGS21680, isoproterenol, forskolin, and BayK 8644 to attenuate preconditioning. Ventricular cells were preconditioned by a 5-min exposure to hypoxia in the presence or absence of the various agents indicated (all were present at 1 μM except BayK 8644 which was 0.1 μM). After replacing with fresh media, the preconditioned cells were exposed to 90 min of hypoxia as did the nonpreconditioned cells. Data were plotted as percent cells killed (*A*) or as the amount of CK released (*B*) and were the mean and standard error of five experiments from five cultures. (● Non-preconditioned; ○ pre-conditioned; △ CG521680; ■ ISO; ▲ Forskolin; □ BayK 8644.)

tion conferred by preconditioning. This notion was supported by the finding that nifedipine (1 μM) abolished the CGS21680-induced attenuation of cardioprotection induced by preconditioning hypoxia ($P > 0.1$ compared to cells preconditioned in the absence of CGS21680 or nifedipine, *t* test) (Fig. 3). To further test the hypothesis, the effect of isoproterenol (0.3 μM), forskolin (1 μM), and BayK 8644 (RBI, Natick, MA) (0.1 μM) on the preconditioning responsiveness was determined. All three agents induced increased calcium influx into the cultured cells (not shown). Both isoproterenol and forskolin stimulated calcium influx by increasing cyclic AMP accumulation (percent increase in cAMP level by isoproterenol, 228±31%, n = 6, ±SE vs. by forskolin, 54.8±9.7%, n = 6) while BayK 8644 activated the calcium channel directly. All three agents, when present during preconditioning hypoxia, caused an increase in percent cells killed and amount of CK released ($P < 0.01$ compared to cells preconditioned in the absence of any agent, ANOVA, and *t* test) (Fig. 3). The deleterious effect of all three agents on the preconditioning response was abolished by 1 μM nifedipine ($P > 0.1$ compared to cells preconditioned in the absence of added agent), indicating that increased calcium entry during preconditioning hypoxia is the mechanism mediating the decreased responsiveness to preconditioning. Neither the calcium entry-promoting agents nor nifedipine had any effect on the extent of injury caused by the 90-min hypoxia when these agents were present only during the preconditioning episode and not during the subsequent 90-min hypoxia (not shown).

Involvement of both adenosine A_1 and A_3 receptors in preconditioning. Blockade of the adenosine A_1 receptor with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) during preconditioning hypoxia did not completely abolish the protection conferred by preconditioning (Fig. 4). Such data are consistent with the notion that an adenosine receptor other than the A_1 subtype, possibly the A_3 receptor, mediates part of the preconditioning response. Whether A_3 receptor is present and whether its activation can simulate preconditioning in the cul-

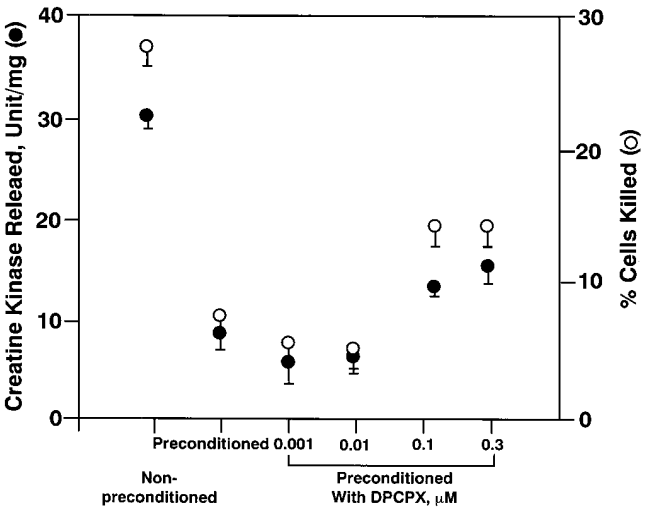

Figure 4. Effect of adenosine A_1 receptor-selective antagonist DPCPX on the protection provided by preconditioning hypoxia. The cultured ventricular cells were exposed to the varying concentrations of DPCPX during the brief preconditioning hypoxia. Media containing DPCPX were replaced with fresh media prior to the 90-min hypoxia. Data were plotted as percent cells killed (○) or as amount of CK released (●) and were the mean and SE of eight experiments from eight cultures. The percent cells killed and the amount of CK released in cells preconditioned in the presence of 0.1 or 0.3 μM DPCPX were less than those in nonpreconditioned cells but were greater than those in cells preconditioned in the absence of DPCPX (one-way ANOVA followed by *t* test, $P < 0.05$).

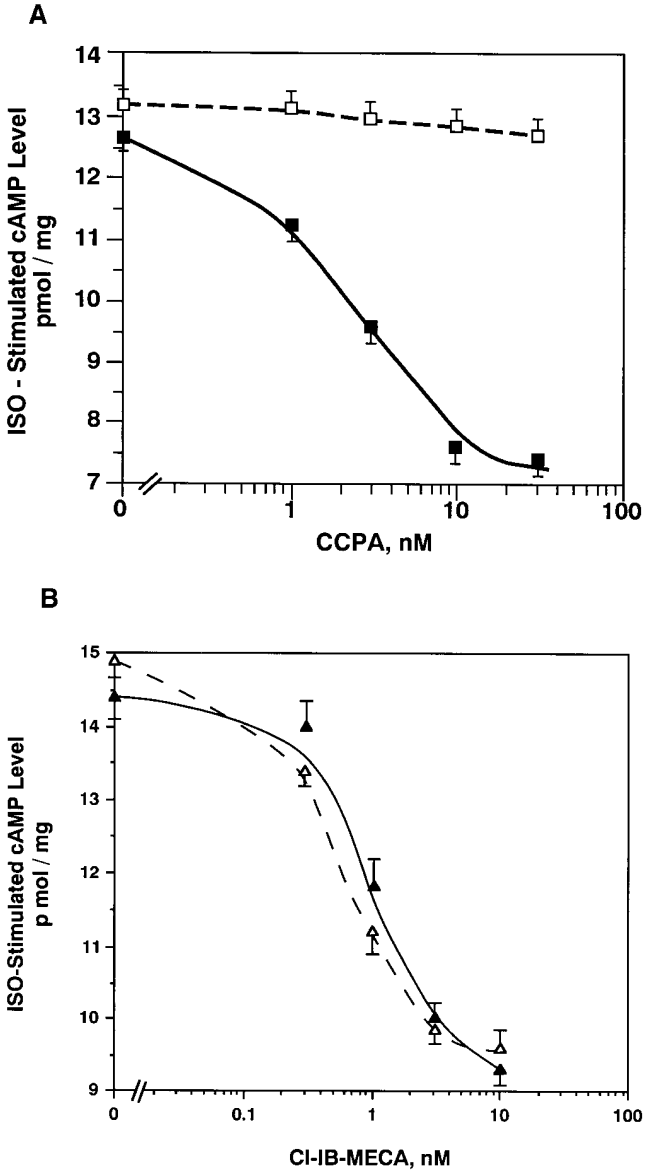

Figure 5. Effects of A_3 receptor-selective agonists on isoproterenol-stimulated cAMP level. Ventricular cells were cultured and the effects of various adenosine agonists and antagonist on the isoproterenol-stimulated cAMP accumulation determined as described in Methods. The levels of cAMP (pmol/mg) were determined in the presence of isoproterenol and varying concentrations of (*A*) CCPA (■) and (*B*) Cl-IB-MECA (▲) or in the presence of isoproterenol, CCPA (□), or Cl-IB-MECA (△) plus 0.1 μM of DPCPX. Data were plotted as cAMP vs. concentrations of the indicated agonists and represented mean±SE of triplicate determinations, which were typical of four other experiments.

tured ventricular cell was determined next. Fig. 5 *A* demonstrated that the A_1-selective agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) caused a dose-dependent inhibition of the isoproterenol-induced stimulation of cAMP accumulation; such inhibition was completely abolished by the A_1-selective antagonist DPCPX. The A_3-selective agonist Cl-IB-MECA (Fig. 5 *B*) or IB-MECA (not shown) was also able to elicit a dose-dependent inhibition of the isoproterenol-induced stimulation of cAMP accumulation, which, however, was not attenuated by DPCPX. Such data suggest that a non-A_1 subtype, likely the A_3 receptor, is present and functional on the cultured ventricular cell.

Fig. 6 *A* showed that prior treatment of the cells with CCPA was able to cause a DPCPX-sensitive preconditioning response in that CCPA pretreatment decreased the extent of CK release or the percent of cells injured during the subsequent 90-min hypoxia. The data indicate that activation of the A_1 receptor could replace preconditioning hypoxia and

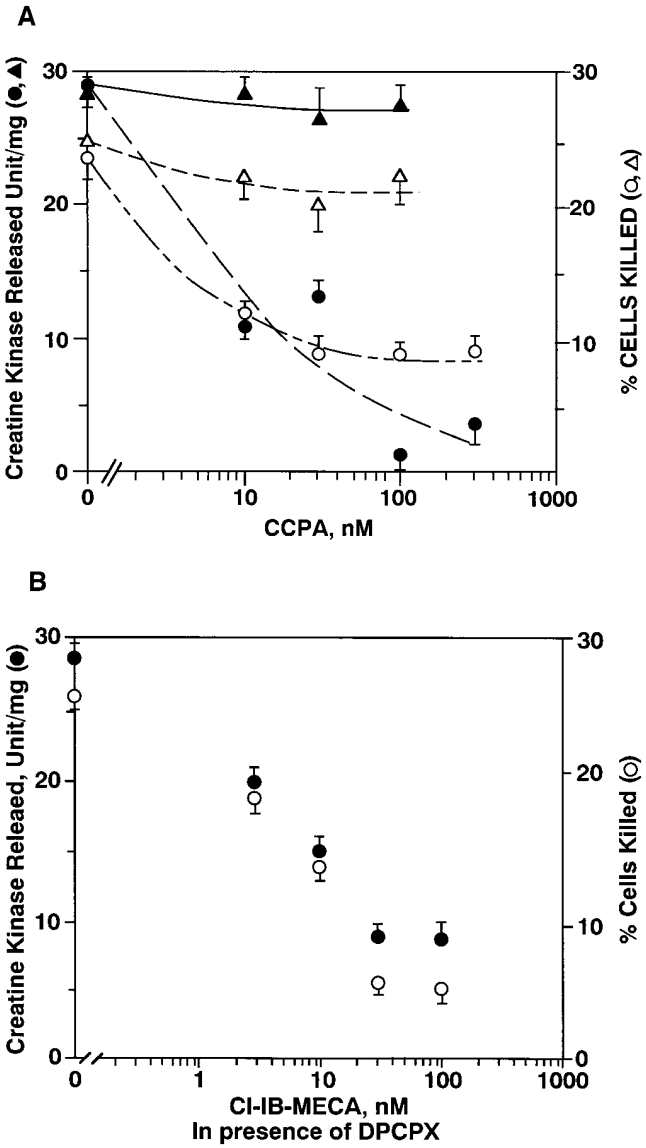

Figure 6. Effects of prior exposure to A_1 and A_3 receptor-selective agonists on the hypoxia-induced cell injury. Cells were preexposed to varying concentrations of (*A*) CCPA in the presence (▲, △) or the absence of 0.1 μM DPCPX (●, ○) or (*B*) Cl-IB-MECA in the presence of 0.1 μM DPCPX (○, ●), then incubated in the presence of fresh media lacking the adenosine analogs for 10 min, and finally exposed to 90 min of hypoxia. Data were plotted as percent cells killed or as amount of CK released vs. varying concentrations of the indicated agonists and were the mean and standard errors of five experiments. The CK released or percent cells killed were higher in cells pretreated with CCPA and DPCPX than in cells pretreated with CCPA alone ($P < 0.01$, *t* test at each CCPA concentration).

simulate preconditioning. Prior exposure of the cells to the A_3 agonists Cl-IB-MECA (Fig. 6 *B*) and IB-MECA (data not shown) was able to induce a DPCPX-insensitive preconditioning response. While DPCPX blocked the CCPA-induced cAMP and preconditioning responses with pA_2 values of 5±2 nM (mean±SE, $n = 3$) and 9±3 nM ($n = 3$), respectively, even 10 μM of DPCPX failed to affect the Cl-IB-MECA-induced cAMP or preconditioning response (not shown). These data suggest that the response mediated by Cl-IB-MECA is the result exclusively of a non-A_1 adenosine receptor, likely the A_3 subtype. The cAMP and preconditioning studies provide further evidence that a functional A_3 receptor is present on the ventricular cell and that its activation can also precondition the ventricular cells against hypoxia-induced injury.

DPCPX, even at 10 μM, was not able to block completely the protective effect of preconditioning hypoxia (not shown). This is similar to the inability of DPCPX to antagonize the Cl-IB-MECA-induced preconditioning response. The nonselective antagonist 8-SPT could block the DPCPX-insensitive protection of preconditioning hypoxia (not shown), similar to the ability of 8-SPT to antagonize the Cl-IB-MECA-induced preconditioning response. The pA_2 value for 8-SPT-mediated inhibition of Cl-IB-MECA-induced preconditioning response (0.5±0.2 μM, $n = 3$, ±SE) was similar to its pA_2 value in inhibiting the Cl-IB-MECA-induced cAMP response (0.2±0.1 μM, $P > 0.1$, paired t test). These data provide further evidence that the DPCPX-insensitive cardioprotective effect, induced by the brief preconditioning hypoxia, is mediated by the A_3 receptor.

Discussion

Brief ischemia, known as preconditioning ischemia, prior to a second sustained ischemia reduces the infarct size in the isolated perfused heart. Although adenosine has been shown to trigger and mediate preconditioning, the role of multiple adenosine receptor subtypes in mediating or modulating preconditioning is not well understood, and the mechanisms modulating responsiveness of ventricular cells to preconditioning stimuli have not been studied. In the present study, ventricular heart cells cultured from chick embryos were developed as a novel myocyte model of preconditioning. A brief hypoxic incubation of the cell was used to simulate preconditioning and a second sustained hypoxic exposure was able to induce injury. The protocol used to precondition the ventricular cells was similar to that employed to precondition the intact heart. The basic characteristics of preconditioning of the myocyte are similar to those of preconditioning of the intact heart (1–4, 11–13). Adenosine triggers as well as mediates the preconditioning effect on the myocytes, similar to its role in preconditioning of the intact heart. In examining the mechanism of adenosine-mediated preconditioning of the myocytes, a number of possibilities can be ruled out. Because these cultured ventricular cells were devoid of sympathetic innervation, it was unlikely that an adenosine-mediated antagonism of catecholamine-stimulated increase in force or rate of myocyte contraction (37) was a mechansim. The lack of circulating blood or vascular cells essentially precluded their involvement in mediating the preconditioning. While the present study does not exclude a role for neuronal, vascular, or blood cells in mediating preconditioning in the intact heart in vivo and while hypoxia in this in vitro model was different from and was not intended to simulate ischemia in vivo, the data provide conclusive evidence that activation of adenosine receptors on ventricular cells can directly precondition the cells against injury. The data suggest that the cultured ventricular cells are an excellent model to study the role of different adenosine receptor subtypes in cardiac myocyte preconditioning.

A novel finding on the action of adenosine in myocyte preconditioning is that activation of the adenosine A_{2a} receptor caused a decrease in the level of protection afforded by preconditioning hypoxia. In fact, blockade of the A_{2a} receptor with the A_{2a}-selective antagonist CSC (27, 33) during preconditioning hypoxia enhanced the protection by preconditioning with a further decrease in the percent cells killed and in the amount of CK released while stimulation with the A_{2a}-selective agonist CGS21680 had the opposite effect. Neither CSC nor CGS21680, when substituted for preconditioning hypoxia, had any protective or adverse effect on the extent of cell injury incurred during the 90-min hypoxia. Such results suggest that adenosine A_{2a} receptor has primarily a modulatory role during preconditioning. Because activation of the A_{2a} receptor resulted in an increase in calcium entry into the cardiac ventricular cell, the question arose regarding whether the increased calcium entry was the mechanism mediating the attenuation of preconditioning. Isoproterenol, acting via the β-adrenergic receptor-G_s-adenylyl cyclase pathway, forskolin, acting via the adenylyl cyclase, and BayK 8644, stimulating the L-type calcium channel directly, all caused a significant increase in the calcium entry. All three agents, when present during preconditioning hypoxia, attenuated the protection by preconditioning. The deleterious effect of all these agents on preconditioning was abolished by nifedipine and correlated with the nifedipine-mediated blockade of calcium entry. Neither isoproterenol, forskolin nor BayK 8644 had any effect on the protection when cells were exposed to these agents for 5 min in place of the preconditioning hypoxia. Although the effect of these agents on the cytosolic calcium level is not known in the present study, the data support the notion that increased calcium entry during the initial brief hypoxia is the mechanism down-modulating the responsiveness of ventricular cells to preconditioning stimuli. Exposure of ventricular cells to nifedipine in the absence of preconditioning hypoxia did not simulate preconditioning, indicating that modulating the level of calcium entry alone could not induce preconditioning.

The ability of the adenosine A_1 receptor agonist R-PIA to simulate preconditioning is consistent with a role of this adenosine receptor in mediating the protective effect of preconditioning. However, the A_1 receptor-selective antagonist DPCPX could block only some of the protective effect of preconditioning when it was present during the preconditioning hypoxia. Since R-PIA can activate both A_1 and A_3 receptor subtypes and since the A_3 receptor is insensitive to blockade by DPCPX, it is possible that the A_3 receptor was also present on these ventricular heart cells and its activation contributed to the protective effect of preconditioning. The present study demonstrates that the A_3 receptor-selective agonists IB-MECA and Cl-IB-MECA (34, 35) were able to cause a marked inhibition of isoproterenol-stimulated cAMP accumulation. While the A_1-selective antagonist DPCPX completely abolished the inhibition of isoproterenol-stimulated cAMP caused by the A_1 agonist CCPA, even 10 μM of DPCPX could not affect the A_3 agonist-mediated inhibition of cAMP. These data suggest that an adenosine receptor subtype

other than the A_1 receptor, likely the A_3 subtype, is also present and can mediate inhibition of isoproterenol-stimulated cAMP accumulation on these ventricular cells. Both A_3 agonists were able to simulate the protective effect of preconditioning hypoxia. The preconditioning effect of A_3 agonists was not blocked by DPCPX while that induced by the A_1 agonist CCPA was completely inhibited by this xanthine, similar to the results obtained in the cAMP study. Previous studies (15, 38) indicated the potential presence of a non-A_1 subtype, possibly an A_3 receptor, on the rabbit myocardium. However, the present study, utilizing a novel ventricular cell model of preconditioning, provides the first conclusive evidence that the adenosine A_3 receptor is present on the ventricular myocyte and that in addition to the A_1 receptor, A_3 receptor activation can also simulate the cardioprotective effect of preconditioning. The possibility that some of these receptors may exert indirect effects via other contaminating cell types is unlikely. This is because the principal nonmyocytes, the fibroblasts, express the A_{2b} receptor and none of the A_1, A_{2a}, or A_3 agonists, at the concentrations used, could cause significant stimulation of the A_{2b} receptor. The data show, for the first time, that a physiologic role of the adenosine A_{2a} receptor is to mediate an antagonistic effect on the preconditioning of ventricular cells and that increased calcium entry during preconditioning stimuli is a novel mechanism capable of attenuating the responsiveness of heart cells to such stimuli. The novel pro- and antiischemic functions of the A_{2a} and A_3 receptors, respectively, should have implications for the treatment of ischemic heart disease. Receptor-selective adenosine analogs such as A_{2a} receptor antagonists or A_3 receptor agonists represent targets for developing new antiischemic pharmacologic agents.

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