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Evidence for deactivation of both ectosolic and cytosolic 5'-nucleotidase by adenosine A1 receptor activation in the rat cardiomyocytes.

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Research Article

Adenosine, an important regulator of many cardiac functions, is produced by ectosolic and cytosolic 5'-nucleotidase. The activity of these enzymes is influenced by several ischemia-sensitive metabolic factors, e.g., ATP, ADP, H+, and inorganic phosphate. However, there is no clear evidence that adenosine itself affects 5'-nucleotidase activity. This study tested whether adenosine decreases the activity of ectosolic and cytosolic 5'-nucleotidase. Cardiomyocytes were isolated from adult male Wistar rats and suspended in the modified Hepes-Tyrode buffer solution. After stabilization, isolated cardiomyocytes were incubated with and without adenosine (10(-9) - 10(-4) M). Ectosolic and cytosolic 5'-nucleotidase activity was decreased by exogenous adenosine (ectosolic 5'-nucleotidase activity, 20.6 +/- 2.3 vs. 8.6 +/- 1.6 mumol/min per 10(6) cells [P < 0.05]; cytosolic 5'-nucleotidase activity, 2.47 +/- 0.58 vs. 1.61 +/- 0.54 mumol/min per 10(6) cells [P < 0.05] at 10(-6) M adenosine) after 30 min. The decrease in ectosolic and cytosolic 5'-nucleotidase activity was inhibited by 8-phenyltheophylline and pertussis toxin, and was mimicked by N6-cyclohexyladenosine, an adenosine A1 receptor agonist. Neither CGS21680C, and A2 receptor agonist, nor cycloheximide deactivated ectosolic and cytosolic 5'-nucleotidase. Thus, we conclude that activation of adenosine A1 receptors is coupled to Gi proteins and attenuates ectosolic and cytosolic 5'-nucleotidase activity in rat cardiomyocytes.

Evidence for Deactivation of Both Ectosolic and Cytosolic 5′-Nucleotidase by Adenosine A_1 Receptor Activation in the Rat Cardiomyocytes

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Abstract

Adenosine, an important regulator of many cardiac functions, is produced by ectosolic and cytosolic 5′-nucleotidase. The activity of these enzymes is influenced by several ischemia-sensitive metabolic factors, e.g., ATP, ADP, H^+, and inorganic phosphate. However, there is no clear evidence that adenosine itself affects 5′-nucleotidase activity. This study tested whether adenosine decreases the activity of ectosolic and cytosolic 5′-nucleotidase. Cardiomyocytes were isolated from adult male Wistar rats and suspended in the modified Hepes-Tyrode buffer solution. After stabilization, isolated cardiomyocytes were incubated with and without adenosine (10^{-9}–10^{-4} M). Ectosolic and cytosolic 5′-nucleotidase activity was decreased by exogenous adenosine (ectosolic 5′-nucleotidase activity, 20.6±2.3 vs. 8.6±1.6 μmol/min per 10^6 cells [$P < 0.05$]; cytosolic 5′-nucleotidase activity, 2.47±0.58 vs. 1.61±0.54 μmol/min per 10^6 cells [$P < 0.05$] at 10^{-6} M adenosine) after 30 min. The decrease in ectosolic and cytosolic 5′-nucleotidase activity was inhibited by 8-phenyltheophylline and pertussis toxin, and was mimicked by N^6-cyclohexyladenosine, an adenosine A_1 receptor agonist. Neither CGS21680C, and A_2 receptor agonist, nor cycloheximide deactivated ectosolic and cytosolic 5′-nucleotidase. Thus, we conclude that activation of adenosine A_1 receptors is coupled to G_i proteins and attenuates ectosolic and cytosolic 5′-nucleotidase activity in rat cardiomyocytes. (*J. Clin. Invest.* 1994. 94:2451–2456.) Key words: G_i protein • 8-phenyltheophylline • N^6-cyclohexyladenosine • CGS21680C • ischemia

Introduction

Adenosine has been reported to modify several key cellular processes in a variety of tissues and organs (1–3). Adenosine relaxes vascular smooth muscles (4), inhibits platelet aggregation (5, 6) and generation of oxygen-derived free radicals from polymorphonuclear leukocytes (7, 8), and attenuates increases in myocardial contractility (9, 10) and the release of norepinephrine from the presynaptic vesicles (11, 12). Adenosine is released from cardiomyocytes, coronary endothelial and smooth muscles, and leukocytes in the heart (2, 3). Of immediate concern is the fact that adenosine is released from cardiomyocytes during ischemia and hypoxia via activation of 5′-nucleotidase, and this enzyme is known to be modulated by ischemia-sensitive metabolic factors, e.g., ATP, ADP, H^+, and inorganic phosphate. Since adenosine is produced by ectosolic and cytosolic 5′-nucleotidase located in the cytoplasm and cellular membrane (2, 3), released adenosine may affect ectosolic and cytosolic 5′-nucleotidase activity. However, no clear consensus exists as to whether adenosine decreases or increases ectosolic and cytosolic 5′-nucleotidase activity. To study whether adenosine modulates 5′-nucleotidase activity, we measured both ectosolic and cytosolic 5′-nucleotidase activity with and without exposure to exogenous adenosine. We also investigated whether a decrease in 5′-nucleotidase activity is receptor mediated using 8-phenyltheophylline and pertussis toxin.

Methods

Materials

Adenosine, N^6-cyclohexyadenosine (CHA)[1], pertussis toxin, 8-phenyltheophylline, and alpha, beta-methyleneadenosine 5′-diphosphate were obtained from Sigma Chemical Co. (St. Louis, MO). CGS21680C was provided by Ciba-Geigy Pharmaceuticals, Inc. (Summit, NJ), and 2′-deoxycofromycin was provided by Yamasa Shoyu Research Laboratories (Choshi, Japan). 5′-Iodotubercidin was obtained from Research Biochems. Inc. (Natick, MA). Antibody of ectosolic 5′-nucleotidase was a gift from Professor Yukio Ikehara, Department of Biochemistry, Fukuoka University School of Medicine. All reagents and chemicals were the highest grade obtainable.

Preparation of cardiomyocytes

Cardiomyocytes were from 97 adult male Wistar rats (200–250 g), as described previously (13). Briefly, the rats were injected with heparin (600 U, i.p.) 30 min before administration of pentobarbital (0.2 mg/g body wt, i.p.). The hearts were removed quickly and perfused in a Langendorff perfusion apparatus, initially with 25 ml of Ca^{2+}-free modified Hepes-Tyrode buffer solution (NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 5 mM, glucose 10 mM, Hepes 20 mM) equilibrated with 95% O_2 and 5% CO_2 at 37°C, and then with a digestive solution (Ca^{2+}-free solution containing 0.06% (wt/wt) crude collagenase and 0.1% fatty acid-free bovine albumin) for 30 min. The ventricle was removed and finely minced with scissors in a Ca^{2+}-containing Hepes-Tyrode solution ($CaCl_2$, 1 mM). Cardiomyocytes were dispersed mechanically by gentle pipetting, filtered through gauze, and suspended in the Ca^{2+}-containing solution. Cells were washed twice with Ca^{2+}-containing solution, and the isolated cells were collected by centrifugation (50 *g*, 1 min) and resuspended in the Ca^{2+}-containing solution

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1. *Abbreviation used in this paper:* CHA, N'-cyclohexyadenosine.

(pH 7.3). Microscopic examination demonstrated that almost all of the isolated cells were cardiomyocytes (2% of the cellular components appeared to be fibroblasts), and that 84±5% of the isolated cardiomyocytes were morphologically (rod-shaped) and metabolically (trypan blue exclusion test) intact.

Some cardiomyocytes were treated with pertussis toxin (10 ng/ml) for 18 h at 37°C (14). Pertussis toxin was activated by incubation at 37°C for 10 min with 10 mM DTT in 100 mM Tris (pH 8.0) and 1 mM EDTA. Viability of cardiomyocytes was not affected by incubation with pertussis toxin. As a control for the pertussis toxin treatment, rat cardiomyocytes were incubated for 18 h without pertussis toxin. Furthermore, we incubated rat cardiomyocytes 18 h with and without cycloheximide. We observed that 80±6% of the incubated isolated cardiomyocytes, with and without pertussis toxin and cycloheximide, were intact.

Experimental protocols

Protocol I. Effects of exogenous adenosine on ectosolic and cytosolic 5′-nucleotidase activity of rat cardiomyocytes. We added 10^{-6} M adenosine to a solution (3 ml) containing $1.0–2.0 \times 10^7$ cells, and observed the temporal changes in ectosolic and cytosolic 5′-nucleotidase activity ($n = 5$). Next, we varied the dose of adenosine from 10^{-9} to 10^{-4} M to determine the dose–response relationship for exogenous adenosine and 5′-nucleotidase activity ($n = 5$ in each dose). Ectosolic and cytosolic 5′-nucleotidase activity was measured 15 min after an exposure to each dose of adenosine because it took 15 min to reach a steady state (Fig. 1). We also observed the temporal changes in the adenosine concentration of the solution containing cardiomyocytes after addition of 10^{-6} M adenosine, and the adenosine concentration of the solution 15 min after the exposure to each dose of adenosine ($10^{-9}–10^{-4}$ M).

Protocol II. Role of adenosine A_1 receptor activation on ectosolic and cytosolic 5′-nucleotidase activity in rat cardiomyocytes. We determined the dose–response relationship between 10^{-9} and 10^{-4} M exogenous adenosine and 5′-nucleotidase activity with concomitant exposures to 8-phenyl-theophylline (10^{-5} M, $n = 5$ in each dose of adenosine), pertussis toxin (10 ng/ml, $n = 5$ in each dose of adenosine), and cycloheximide (0.2×10^{-6} g/ml, $n = 5$ in each dose of adenosine). We also measured ectosolic and cytosolic 5′-nucleotidase activity 15 min after an exposure to each dose of adenosine during concomitant treatment with pertussis toxin. To confirm the effect of pertussis toxin on G_i proteins in rat cardiomyocytes, we measured cyclic AMP contents before and 15 min after exposure to 10^{-8} and 10^{-6} M isoproterenol with and without adenosine (10^{-6} M). If G_i protein is deactivated by pertussis toxin, adenosine can not attenuate the increases in cyclic AMP content of cardiomyocytes. Second, we added 10^{-6} M CHA to a solution containing $1.0–2.0 \times 10^7$ rat cardiomyocytes. We observed the temporal changes in the ectosolic and cytosolic 5′-nucleotidase activity after an exposure to CHA 10^{-6} M ($n = 5$). We determined the dose–response relationship between CHA ($10^{-9}–10^{-4}$ M) and ectosolic and cytosolic 5′-nucleotidase activity ($n = 6$ in each dose) as well as the dose–response relationship between CGS21680C ($10^{-9}–10^{-4}$ M) and ectosolic and cytosolic 5′-nucleotidase activity. CHA and CGS21680C are agonists of adenosine A_1 and A_2 receptors, respectively. Finally, to test the possibility that ectosolic 5′-nucleotidase is removed from the cellular surface during exposure to adenosine, we performed immunoblotting of ectosolic 5′-nucleotidase treated with and without adenosine (10^{-6} M) for 30 min.

Adenosine and cyclic AMP measurements

To measure temporal changes in adenosine release from cardiomyocytes (15, 16), we obtained the medium of the suspension of cardiomyocytes by centrifugation with 50 *g* for 1 min. We added 10 mM EDTA, and 10% TCA in the medium to inhibit degradation of adenosine and 5′-AMP. TCA was removed by water-saturated ether. After centrifugation (1,000 *g*), the supernatant was collected and the adenosine content was determined by radioimmunoassay.

Adenosine in the solution (100 μl) was succinylated by 100 μl of dioxane containing succinic acid anhydride and trimethyamine. After a

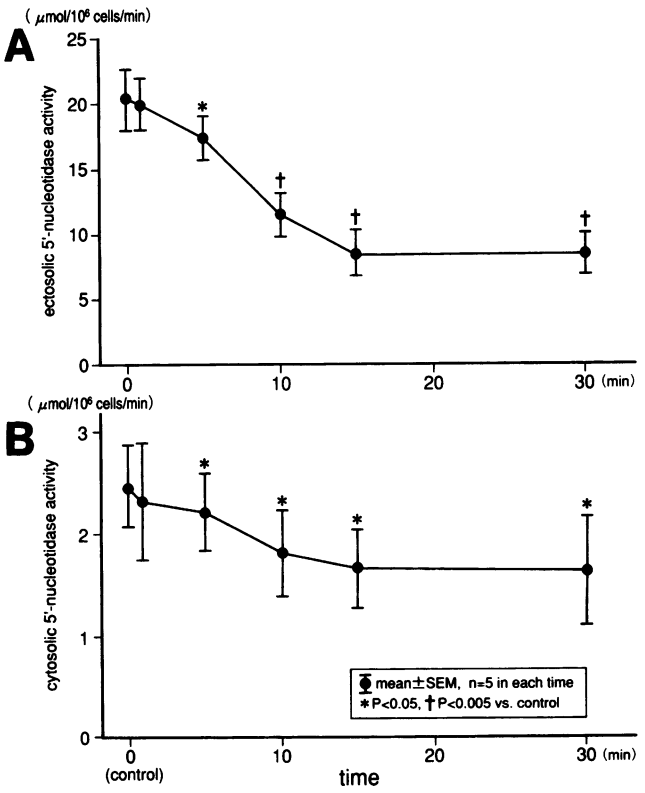

Figure 1. Temporal changes in ectosolic (*A*) and cytosolic (*B*) 5′-nucleotidase activity in rat cardiomyocytes following exposure to adenosine 10^{-6} M. Adenosine 10^{-6} M reduced both ectosolic and cytosolic 5′-nucleotidase activity.

20-min incubation, the mixture was kept in a cold incubation, and was diluted with 100 μl of adenosine 2′, 3′-O-disuccinyl-3-[^{125}I]-iodotyrosine methyl ester (0.5 pmol), and 100 μl of diluted antiadenosine serum. The mixture was kept in a cold water (4°C) bath for 18 h, and the second antibody solution (500 μl goat anti–rabbit immunoglobulin G antiserum) was added. After incubation at 4°C for 1 h, the unreacted materials were removed by centrifugation at 2,500 *g* at 4°C for 20 min. The radioactivity remaining in the tube was counted using a gamma counter. Since it was possible that unidentified products of adenosine metabolism or the substrates other than adenosine in the supernatant interfere with the immunoassay of adenosine, we tested to see if immunoreactive adenosine can be removed by incubation with adenosine deaminase (10 U/ml). Measured adenosine concentration in the supernatant was decreased to 5±3%, indicating that this assay technique provides the adenosine concentration with high specificity.

The method of the measurement of cyclic AMP concentration in tissues has been previously described (17). After exposures to adenosine and isoproterenol with and without pertussis toxin treatment, cardiomyocytes were frozen in liquid nitrogen, and immediately stored at −80°C in liquid nitrogen. The frozen tissue was powdered, and homogenized at 4°C in 1 ml of ice-cold 10% TCA, centrifuged at 2,500 *g* for 20 min. The supernatant fluid was removed and extracted three times with 3 ml of diethyl ether saturated with water, and stored in the freezer (−80°C). The cyclic AMP concentration in the supernatant fluid was measured by the radioimmunoassay method (17). Briefly, 100 μl of dioxane-triethylamine mixture containing succinic acid anhydride succinylated cyclic AMP in the supernatant (100 μl). After a 10-min incubation, the reaction mixture was added to 800 μl of 0.3 M imidazole buffer (pH 6.5). 100 μl of succinyl cyclic AMP tyrosine methyl ester iodinated with ^{125}I (15,000–20,000 cpm in an amount $< 10^{-14}$ M) was added to the assay mixture containing 100 μl of the supernatant and 100 μl of diluted antisera in the presence of chloramine T; the mixture was kept

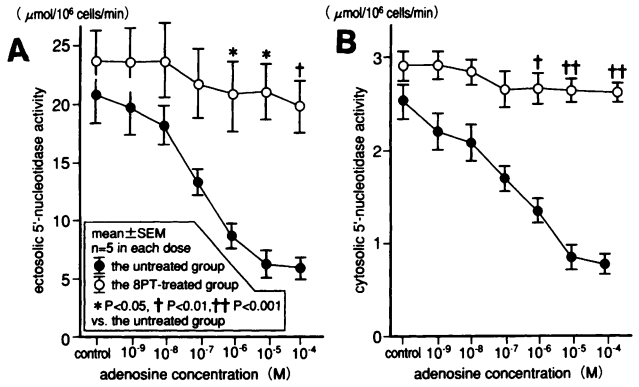

Figure 2. The dose–response relationship between the concentration of extracellular adenosine and ectosolic (*A*) and cytosolic (*B*) 5′-nucleotidase activity with and without concomitant exposure to 8-phenyltheophylline in rat cardiomyocytes. Rat cardiomyocytes were exposed to each dose of adenosine for 15 min because it took 15 min to reach a steady state of the activity of ectosolic and cytosolic 5′-nucleotidase as is evident in Fig. 1. The reduction in both ectosolic and cytosolic 5′-nucleotidase activity due to adenosine exposure was blunted by treatment with 8-phenyltheophylline.

at 4°C for 24 h. A cold solution of dextran-coated charcoal (500 μl) was added to the mixture in an ice-cold water bath. The charcoal was spun down, and the radioactivity of 0.5 ml of the supernatant was counted using a gamma counter.

Measurement of 5′-nucleotidase activity

Once the experiments were completed, the rat cardiomyocytes were homogenized for 5 min in 10 vol of ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-potassium hydroxide (Hepes-KOH) buffer (pH 7.4) containing 0.25 M sucrose, 1 mM $MgCl_2$, and 1 mM mercaptoethanol. The crude homogenate was strained through a double-layered nylon sieve and again homogenized for 1 min. To prepare a crude membrane fraction, part of the homogenate was centrifuged at 1,000 *g* for 10 min. The resultant pellet was washed three times and resuspended in Hepes-KOH buffer. To prepare the cytosolic fraction, the remainder of the homogenate was centrifuged at 3,000 *g* for 10 min, and the supernatant was centrifuged again at 200,000 *g* for 1 h. The membrane and cytosolic fractions were dialyzed at 4°C for 4 h against 10 mM Hepes-KOH (pH 7.4) containing 1 mM $MgCl_2$, 1 mM mercaptoethanol, and 0.01% activated charcoal, and divided into aliquots which were frozen immediately and stored at −80°C.

The activity of 5′-nucleotidase was assessed by the enzymatic assay technique (18). 5′-Nucleotidase activity of the membrane and cytosolic fractions were defined as ectosolic and cytosolic 5′-nucleotidase activity, respectively. When cytosolic 5′-nucleotidase activity was measured, alpha,beta-methyleneadenosine 5′-diphosphate (50 μM), an inhibitor of ectosolic 5′-nucleotidase, was added to inhibit contaminated ectosolic 5′-nucleotidase.

Immunoblotting of ectosolic 5′-nucleotidase

The supernatants obtained for the measurement of ectosolic 5′-nucleotidase activity were used for the immunoblotting of ectosolic 5′-nucleotidase. The proteins of the supernatants were separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinyldifluoride (Millipore, Bedford, MA) for immunoblotting with anti-ectosolic 5′-nucleotidase antibody (rabbit serum, 1/1,000 dilution), and subsequently revealed by alkaline phosphatase–conjugated goat anti–rabbit IgG as a secondary antibody (1/3,000 dilution, Bio-Rad Laboratories, Richmond, CA) using 5% powdered skim milk in TBS as a blocking agent. Alkaline phosphatase activity was detected using bromochloroindolyl phosphate/nitro blue tetrazolium as a substrate.

Statistical analysis

Statistical analysis was performed using paired and unpaired *t* tests (19). Repeated measures of ANOVA followed by modified Bonferonni's multiple comparison were also performed to evaluate differences in the time course and dose–response curve between the two groups. All values were expressed as means±SEM; $P < 0.05$ was considered significant.

Results

Ectosolic 5′-nucleotidase activity in rat cardiomyocytes decreased from 20.6±2.3 to 8.6±1.6 μmol/min per 10^6 cells after 30 min of exposure to exogenous adenosine 10^{-6} M: It began to decrease within 5 min after exposure to adenosine, and thereafter became stable at 15 min (Fig. 1*A*). Cytosolic 5′-nucleotidase activity was also decreased by exposure to adenosine 10^{-6} M (Fig. 1 *B*). Increased concentrations of exogenous adenosine decreased both ectosolic and cytosolic 5′-nucleotidase activity (Fig. 2). Table I shows the adenosine concentrations of the solution containing rat cardiomyocytes after 15 min of exposure to each dose of exogenous adenosine. The degradation and uptake of the added adenosine were less than 5% for 15 min in each dose of adenosine.

We next investigated whether the decreases in ectosolic and cytosolic 5′-nucleotidase activity were adenosine A_1 receptor mediated. First, 8-phenyltheophylline blunted the decreases in ectosolic and cytosolic 5′-nucleotidase activity due to exposures to adenosine at concentrations from 10^{-9} to 10^{-4} M (Fig. 2). We also tested whether CHA and CGS21680C mimic the decreases in ectosolic and cytosolic 5′-nucleotidase activity seen with exposure to exogenous adenosine. Exposure to CHA decreased ectosolic and cytosolic 5′-nucleotidase activity in rat cardiomyocytes in a dose-dependent manner (Figs. 3 and 4). In contrast, CGS21680C did not decrease either ectosolic or cytosolic 5′-nucleotidase activity (Fig. 4). Third, we tested to see if pertussis toxin, which inhibits G_i proteins, blunts the decrease in ectosolic and cytosolic 5′-nucleotidase activity due

Table I. Changes in Adenosine Concentration (M) of the Solution Containing Rat Cardiomyocytes after 15 Min of Exposure to Each Dose of Adenosine

Adenosine exposure	Control	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Before exposure	6.00±0.29 ×10^{-9}	6.91±1.41 ×10^{-9}	8.04±2.25 ×10^{-9}	7.43±2.25 ×10^{-9}	6.32±0.98 ×10^{-9}	7.74±0.73 ×10^{-9}	7.62±1.56 ×10^{-9}
15 min after exposure of each dose of adenosine	6.92±0.32 ×10^{-9}	7.61±1.92 ×10^{-9}	1.49±0.002 ×10^{-8}	0.961±0.011 ×10^{-7}	0.954±0.021 ×10^{-6}	0.952±0.025 ×10^{-5}	0.956±0.019 ×10^{-4}

Values (M) are mean±SEM.

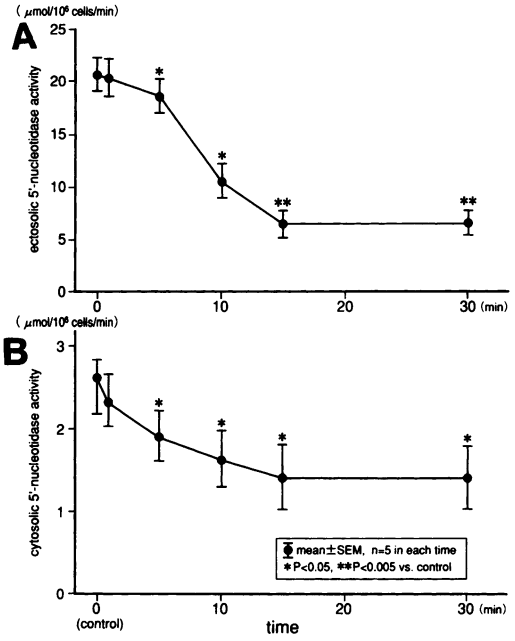

Figure 3. Temporal changes in ectosolic (*A*) and cytosolic (*B*) 5′-nucleotidase activity following exposure to CHA 10^{-6} M. CHA 10^{-6} M reduced both ectosolic and cytosolic 5′-nucleotidase activity.

to adenosine exposure. Pertussis toxin (10 ng/ml) markedly attenuated the adenosine-induced inhibition of the increases in cyclic AMP content due to isoproterenol (cyclic AMP content [μmol/10^6 cells] due to 10^{-8}, 10^{-7}, and 10^{-6} M of isoproterenol: 2.03±0.17, 5.98±0.38, 8.47±0.50, and 8.60±0.43 in the untreated condition; 2.17±0.24, 3.18±0.34, 4.28±0.27 and 4.77±0.24 in the adenosine-treated condition; 2.25±0.34, 5.77±0.58, 8.70±0.72 and 8.90±0.77 in the adenosine-treated condition with pertussis toxin, $P < 0.001$ vs. the untreated condition). The treatment with pertussis toxin attenuated the decreases in ectosolic and cytosolic 5′-nucleotidase activity due to exposure to adenosine concentrations from 10^{-9} to 10^{-4} M (Fig. 5).

We further examined whether the inhibition of synthesis

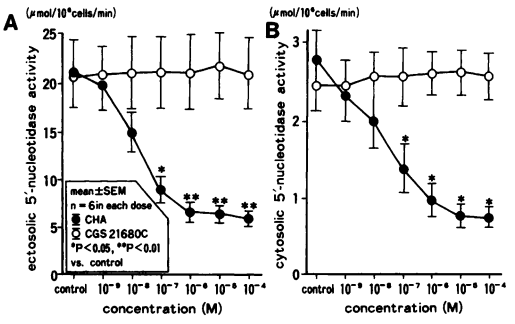

Figure 4. The dose–response relationship between the concentrations of extracellular CHA (an adenosine A_1 receptor agonist) and CGS21680C (an adenosine A_2 receptor agonist), and ectosolic (*A*) and cytosolic (*B*) 5′-nucleotidase activity in rat cardiomyocytes. Both ectosolic and cytosolic 5′-nucleotidase activity was reduced when the concentration of CHA was increased, however, neither ectosolic nor cytosolic 5′-nucleotidase activity changed when the dose of CGS21680C was increased.

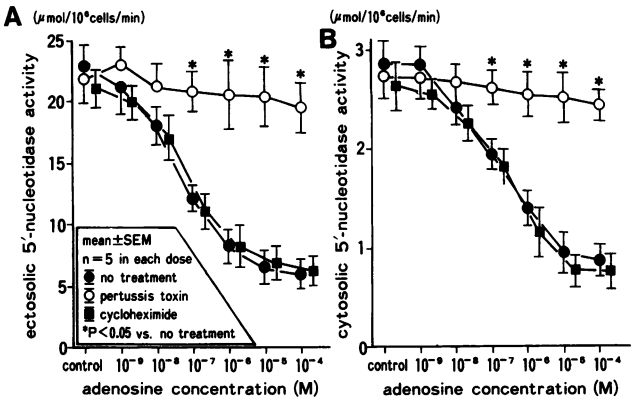

Figure 5. The dose–response relationships between the extracellular concentration of adenosine, and ectosolic (*A*) and cytosolic (*B*) 5′-nucleotidase activity during concomitant exposures to pertussis toxin and cycloheximide in rat cardiomyocytes. The increase in both ectosolic and cytosolic 5′-nucleotidase activity produced by exposure to a progressively greater concentration of adenosine was not blunted by cycloheximide, but was blunted by treatment with pertussis toxin. The rat cardiomyocytes were incubated for 18 h without any pharmacological intervention (the no treatment group) as well as the treatments with pertussis toxin and cycloheximide.

of ectosolic and cytosolic 5′-nucleotidase is involved in the deactivation of ectosolic and cytosolic 5′-nucleotidase. Cycloheximide did not affect the adenosine-induced deactivation of ectosolic and cytosolic 5′-nucleotidase (Fig. 5). The immunoblotting of untreated- and adenosine (10^{-6} M)-treated cardiomyocytes revealed that the amount of ectosolic 5′-nucleotidase is not altered by exposure to adenosine (Fig. 6). These results indicate that (*a*) exogenous adenosine attenuates ectosolic and cytosolic 5′-nucleotidase activity, (*b*) the adenosine-induced decreases in ectosolic and cytosolic 5′-nucleotidase activity are A_1-receptor-mediated and coupled with G_i proteins, and (*c*) neither inhibition of protein synthesis nor removal of ectosolic 5′-nucleotidase from the cellular membrane is involved in the adenosine-induced decreases in 5′-nucleotidase activity.

Discussion

Activation of myocardial adenosine A_1 receptors and ectosolic and cytosolic 5′-nucleotidase activity. Ectosolic and cytosolic

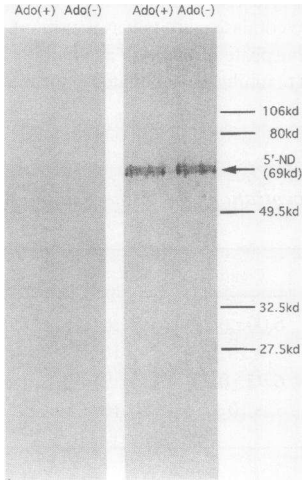

Figure 6. Immunoblotting of ectosolic 5′-nucleotidase with and without adenosine exposure (10^{-6} M). The number of cariomyocytes in the four lanes was comparable (5.3, 5.6, 5.5, and 5.4 × 10^4 cells from the left to the right lanes). Although there were marked differences in ectosolic 5′-nucleotidase activity between the untreated (Ado −) and adenosine-treated (Ado +) cardiomyocytes (22.5 vs. 9.7 μmol/min per 10^6 cells), adenosine exposure did not affect the amount of ectosolic 5′-nucleotidase. 5′-ND, ectosolic 5′-nucleotidase.

5′-nucleotidase are thought to be primarily responsible for the synthesis of adenosine in the heart (1–3), and both enzymes are affected by a number of metabolic factors (20–22). Ectosolic 5′-nucleotidase is strongly inhibited by ATP and ADP (20, 21), whereas cytosolic 5′-nucleotidase activity is activated by ATP and ADP, and inhibited by inorganic phosphate (22, 23). This study shows that adenosine inhibits both ectosolic and cytosolic 5′-nucleotidase. This phenomenon may constitute a negative feedback mechanism or end-product inhibition for adenosine production in cardiomyocytes.

The A_1 adenosine receptor–mediated signal transduction in the rat cardiomyocytes is necessary to downregulate 5′-nucleotidase activity. Adenosine is reported to increase the content of inositol 1,4,5-trisphosphate through increases in diacylglycerol (24), suggesting that protein kinase C may deactivate ectosolic and cytosolic 5′-nucleotidase. However, we have preliminarily reported that protein kinase C rather activates 5′-nucleotidase in rat cardiomyocytes (25). There may be a possibility that ectosolic 5′-nucleotidase is removed from the cellular surface; however, this study may deny that possibility because there are no differences of amount of ectosolic 5′-nucleotidase revealed by the immunoblotting of ectosolic 5′-nucleotidase with and without adenosine exposure. Thus, this study did not determine the exact mechanism by which adenosine A_1 receptor activation decreases ectosolic and cytosolic 5′-nucleotidase activity.

It is possible that activation of G_i proteins may change the cellular membrane adjacent to ectosolic 5′-nucleotidase and promote the internalization of this enzyme (26). Allosteric factors may be partially responsible for the activation of 5′-nucleotidase, and activation of G_i proteins may modify this allosteric effect.

Reduction of ectosolic and cytosolic 5′-nucleotidase activity and adenosine release from rat cardiomyocytes. This study does not necessarily indicate that adenosine release is attenuated when adenosine A_1 receptors of cardiomyocytes are activated, because 5′-nucleotidase is not the sole determinant of adenosine release. There are two major pathways for adenosine synthesis: The enzymatic dephosphorylation of 5′-adenosine monophosphate (5′-AMP) by 5′-nucleotidase, and the hydrolysis of *S*-adenosylhomocysteine hydrolase (27–29). 5′-Nucleotidase exists in both cellular membrane and cytoplasm, with both being able to produce adenosine (30–32). Our study did not establish whether one pathway is more important, and several lines of evidence suggest that both are essential for the production of adenosine in the hypoxic cardiomyocytes (23, 29–32).

The activity of adenosine kinase and adenosine deaminase as potential determinants of adenosine production in rat cardiomyocytes also needs to be considered (28). Schrader et al. (31) have reported that adenosine kinase contributes to adenosine production in cardiomyocytes. However, the present study does not prove that activation of G_i proteins changes the activity of adenosine kinase and deaminase. This idea needs to be elucidated in further studies.

Pathophysiological relevances in the heart. The finding in this report enhances our understanding of coronary vascular physiology and cardiac pathophysiology. The balance between the amounts of extracellular adenosine acting to produce coronary vasodilation and intracellular adenosine available for ATP synthesis is essential in regulating coronary vascular tone and myocardial metabolism during ischemia and reperfusion. The present results may establish a new regulatory mechanism for myocardial cellular homeostasis in which released adenosine attenuates adenosine production by decreasing 5′-nucleotidase activity, which in turn spares intracellular adenosine for incorporation into ATP. This finding may explain the interesting observation (33) that norepinephrine infusion increases adenosine release with overshoot, and that adenosine concentration eventually returns to the value between the baseline and the peak values. Decreased 5′-nucleotidase activity secondary to an increase in the adenosine concentration may partially explain this phenomenon.

In summary, our results hint that the relationship between endogenous adenosine and deactivation of 5′-nucleotidase plays an important role in the switching mechanisms governing storage and release of adenosine for cardioprotection during ischemia.

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References

1. Berne, R. M. 1980. The role of adenosine in the regulation of coronary blood flow. *Circ. Res.* 47:807–813.

2. Hori, M., and M. Kitakaze. 1991. Adenosine, the heart, and coronary circulation. *Hypertension.* 19:565–574.

3. Kitakaze, M., M. Hori, and T. Kamada. 1993. Interaction between adenosine and alpha-adrenoceptor activity and its role of ischemia and reperfusion injury. *Cardiovasc. Res.* 27:18–27.

4. Berne, R. M., H. R. Winn, R. M. Knabb, S. W. Ely, and R. Rubio. 1983. Blood flow regulation by adenosine in heart, brain and skeletal muscle. *In* Regulatory Function of Adenosine. R. M. Berne, T. W. Rall, and R. Rubio, editors. Martinus Nijhoff Publishers, Boston, MA. 293–317.

5. Agarwal, K. C. 1987. Adenosine and platelet function. *In* Role of Adenosine in Cerebral Metabolism and Blood Flow. V. Stefanovich and I. Okayuz-Baklouti, editors. VNU Science Press, Utrecht, The Netherlands. 107–124.

6. Kitakaze, M., M. Hori, H. Sato, S. Takashima, M. Inoue, A. Kitabatake, and T. Kamada. 1991. Endogenous adenosine inhibits platelet aggregation during myocardial ischemia in dogs. *Circ. Res.* 69:1402–1408.

7. Cronstein, B. N., R. I. Levin, J. Belanoff, G. Weissmann, and R. Hirschhorn. 1986. Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J. Clin. Invest.* 78:760–770.

8. Engler, R. 1987. Consequences of activation and adenosine-mediated inhibition of granulocytes during myocardial ischemia. *Fed. Proc.* 46:2407–2412.

9. Schrader, J., G. Baumann, and E. Gerlach. 1977. Adenosine as inhibitor of myocardial effects of catecholamines. *Pfluegers Arch. Eur. J. Physiol.* 372:29–35.

10. Belardinelli, L., and G. Isenberg. 1983. Actions of adenosine and isoproterenol on isolated mammalian ventricular myocytes. *Circ. Res.* 53:287–297.

11. Hedqvist, P., and B. B. Fredholm. 1979. Inhibitory effect of adenosine on adrenergic neuroeffector transmission in the rabbit heart. *Acta Physiol. Scand.* 105:120–122.

12. Rechardt, G., W. Waas, R. Kranzhofer, E. Mayer, and A. Schomig. 1987. Adenosine inhibits exocytotic release of endogenous noradrenaline in rat heart: a protective mechanism in early myocardial ischemia. *Circ. Res.* 61:117–123.

13. Iwakura, K., M. Hori, Y. Watanabe, A. Kitabatake, E. J. Cragoe, Jr., H. Yoshida, and T. Kamada. 1990. Alpha$_1$-adrenoceptor stimulation increases intracellular pH and Ca^{2+} in cardiomyocytes through Na^+/H^+ and Na^+/Ca^{2+} exchange. *Eur. J. Pharmacol.* 186:29–40.

14. Katada, T., T. Amano, M. Ui. 1982. Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. *J. Biol. Chem.* 257:3739–3746.

15. Kitakaze, M., M. Hori, J. Tamai, K. Iwakura, Y. Koretsune, T. Kagiya, K. Iwai, A. Kitabatake, M. Inoue, and T. Kamada. 1987. Alpha$_1$-adrenoceptor activity regulates release of adenosine from the ischemic myocardium in dogs. *Circ. Res.* 60:631–639.

16. Yamane, R., T. Nakamura, E. Matsuura, H. Ishige, and M. Fujimoto. 1991. A simple and sensitive radioimmunoassay for adenosine. *J. Immunol.* 12:501–519.

17. Honma, M., T. Satoh, J. Takezawa, and M. Ui. 1977. An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small-volume samplings from blood and tissue. *Biochem. Med.* 18:257–273.

18. Smith, K., H. H. Vaton, G. J. Race, D. L. Paulson, H. C. Urshel, and J. T. Mallams. 1965. Serum 5′-nucleotidase in patients with tumor in the liver. *Cancer.* 19:1281–1285.

19. Winer, B. J. 1982. Statistical Principles in Experimental Design. Second edition. McGraw-Hill Book Co., New York. 1–907.

20. Sullivan, J. M., and J. B. Alpers. 1971. In vivo regulation of rat heart 5′-nucleotidase by adenine nucleotidase and magnesium. *J. Biol. Chem.* 246:3057–3063.

21. Luzio, J. P., E. M. Bailyes, C. Van Pottlesberghe, and H. G. Hers. 1986. The properties, structure, function, intracellular localization and movement of hepatic 5′-nucleotidase. *In* Cellular Biology of Ectoenzymes. G. W. Kreuzberg, editor. Springer-Verlag, Berlin/Heidelberg/New York. 89–116.

22. Van der Berghe, G., C. Van Pottelsberghe, and H. G. Hers. 1977. A kinetic study of soluble 5′-nucleotidase of rat liver. *Biochem. J.* 162:611–616.

23. Lowenstein, J. M., M. K. Yu, and Y. Naito. 1983. Regulation of adenosine metabolism by 5′-nucleotidase. *In* Regulatory Function of Adenosine. R. M. Berne, T. W. Rall, and R. Rubio, editors. Martinus Nijhoff Publishers, Boston, MA. 117–129.

24. Kohl, C., B. Linck, W. Schmitz, H. Scholz, J. Scholz, and M. Toth. 1990. Effect of carbachol and (−)-N^6 phenylisopropyladenosine in myocardial inositol phosphate content and force of contraction. *J. Pharmacol.* 101:829–834.

25. Kitakaze, M., and A. Kitabatake. 1991. Increased 5′-nucleotidase activity caused by protein kinase C enhances adenosine production in hypoxic cardiomyocytes of rat. *Circulation.* 84:II–620. (Abstr.)

26. Widnell, C. C., Y. J. Schneider, B. Pierre, P. Bandhurn, and A. Trouet. 1982. Evidence for a continual exchange of 5′-nucleotidase between the cell surface and cytoplasmic membrane in cultured rat fibroblasts. *Cell.* 28:61–70.

27. Achterberg, P. W., de P. Tombe, E. Harmsen, and de J. W. Jong. 1985. Myocardial *S*-adenosylhomocysteine hydrolase is important for adenosine production during normoxia. *Biochim. Biophys. Acta.* 840:393–400.

28. Lloyd, H. G. E., and J. Schrader. 1987. The importance of the transmethylation pathway for adenosine metabolism in the hearts. *In* Topics and Perspectives in Adenosine Research. E. Gerlach and B. F. Becker, editors. Springer-Verlag. Berlin/Heidelberg. 199–207.

29. Sparks, H. V., Jr., and H. Bardenheuer. 1986. Regulation of adenosine formation by the hearts. *Circ. Res.* 58:193–201.

30. Newby, A. C., Y. Worku, and P. Meghji. 1987. Critical evaluation of role of ecto- and cytosolic 5′-nucleotidase in adenosine formation. *In* Topics and Perspective in Adenosine Research. E. Gerlach and B. F. Becker, editors. Springer-Verlag, Berlin/Heidelberg. 155–168.

31. Schrader, J., M. Borst, M. Kelm, T. Smolenski, and A. Deussen. 1991. Intra- and extracellular formation of adenosine by cardiac tissue. *In* Role of Adenosine and Adenine Nucleotides in the Biological System. S. Imai and M. Nakazawa, editors. Elsevier Science Publisher, Amsterdam. 261–270.

32. Newby, A. C., C. A. Holmquist, J. Illingworth, and J. D. Pearson. 1983. The control of adenosine concentration in polymorphonuclear leukocytes, cultured heart cells and isolated perfused heart from the rat. *Biochem. J.* 214:317–323.

33. DeWitt, D. F., R. D. Wangler, C. I. Thompson, and H. V. Sparks. 1983. Phasic release of adenosine during steady-state metabolic stimulation in the isolated guinea pig heart. *Circ. Res.* 53:636–643.