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Assessment of the cytoprotective role of adenosine in an in vitro cellular model of myocardial ischemia

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Abstract

This work aimed to detect functional adenosine receptors in isolated rat cardiomyocytes and to study the influence of stimulation of these receptors in an in vitro model of ischemia. Cultures of cardiomyocytes were prepared from newborn rat ventricles. The contractions were photometrically monitored. In this preparation, adenosine induced a positive chronotropic response. This effect was reproduced by CGS 21680 (2-(4-[2-carboxyethyl]-phen-ethyl-amino) adenosine-5′*N*-ethylunosamide), a specific adenosine A_2 receptor agonist, and antagonized by DMPX (3,7-dimethyl-1-propargylxanthine), an adenosine A_2 receptor antagonist. However, R-PIA (*R*-N^6-(2-phenylisopropyl)-adenosine; a specific adenosine A_1 receptor agonist) induced a negative chronotropic effect that was abolished by its corresponding adenosine A_1 antagonist DPCPX (1,3-dipropyl-8-cyclo-pentyl-adenosine). Substrate-free hypoxia, as simulation of ischemia, induced a progressive decrease and then arrest of spontaneous cell contractions. The spontaneous rhythmic contractile activity was restored during reoxygenation following simulated ischemia. Adenosine A_1 receptor stimulation with R-PIA induced a decrease of hypoxia-induced damage. This effect was antagonized by DPCPX, an adenosine A_1 receptor antagonist. Conversely, the cells treated with CGS 21680 did not display complete recovery after reoxygenation. In addition, this effect was abolished by DMPX, since the cells recovered normal function after reoxygenation. To conclude, it appeared that cardiomyocytes possess both functional adenosine A_1 and A_2 receptors and that only the activation of adenosine A_1 receptor had a cytoprotective effect against simulated ischemia-induced cardiac cell injury.

Keywords: Cardiomyocyte; Cell culture; Electrophysiology; Cell contraction; Adenosine receptor; Simulated ischemia-reperfusion; (Rat)

1. Introduction

Adenosine is an adenylic nucleotide metabolite widely distributed throughout the human body and exerts a wide range of regulatory effects. Adenosine membrane receptors belong to the extensive family of receptors bound to protein G and are glycoproteins with seven transmembrane domains. Adenosine receptors mediate various cardiovascular effects (Linden, 2001), but the mechanisms underlying the different physiological effects are not yet completely clarified.

Besides its regulatory role in the cardiovascular system, adenosine is also involved in the response of the myocardium to metabolic stress. The production and the release of adenosine by the myocardium are increased either when the myocardial O_2 demand is increased, such as during an episode of increased load, or when the O_2 supply decreases, such as during ischemia. This endogenous adenosine production presumably protects the heart against the noxious effects of adrenergic activation (Khandoudi et al., 1994), because this anti-adrenergic effect tends to reduce the O_2 demand of the myocardium. The cardioprotective potency of adenosine has been also detected in rat cardiomyocytes, where damage induced by hypoxia and reoxygenation is alleviated by stimulating adenosine liberation via the activation of 5′-nucléotidase (Kitakaze et al., 1996; Sommerschild et al., 1997). Suto et al. (2000) also indicated that the high sensitivity of adenosine could also play a role in the ischemic tolerance of newborn hearts. For these reasons, adenosine and its receptors have been the subject of numerous studies focusing on myocardial ischemia-reperfusion.

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Adenosine is actively produced during ischemia, as a result of the degradation of ATP. The suggested beneficial effects of adenosine during ischemia raised also the question of a possible role of adenosine in ischemic preconditioning. Preconditioning, described for the first time by Murry et al. (1986), is defined as an adaptation of the myocardium to the ischemic stress induced by short periods of ischemia before a longer period of ischemia. The use of adenosine as initiator of preconditioning before angioplasty has led to encouraging results (Leesar et al., 1997). However, in spite of the large number of studies supporting the protective role of adenosine, the suggested mechanisms differ according to the experimental model used. In particular, the type of adenosine receptors involved in preconditioning is still debated. The predominant role of adenosine A_1 receptors was suggested by several studies (Liang, 1996; Pisarenko et al., 1997; Baxter et al., 1997), but the contribution of adenosine A_3 receptors has been also suspected (Wang et al., 1997). Other reports indicated that stimulation of both adenosine A_1 and A_2 receptors by the corresponding selective adenosine receptor agonists could also protect the canine heart from ischemic injury (McVey et al., 1999). These two adenosine receptors might also be involved in protecting the rat myocardium against ischemia-reperfusion (Lozza et al., 1997). Fewer studies have been conducted with isolated cardiac muscle cells. Nevertheless, it has been recently suggested that adenosine receptor agonists in rat cardiomyocytes may attenuate myocyte injury during hypoxia (Safran et al., 2001).

Therefore, the purpose of the present study was to determine the presence of adenosine receptors in isolated rat cardiomyocytes and to evaluate the influence of the activation of these receptors on cardiomyocyte functional damage caused by in vitro simulated ischemia-reperfusion. Briefly, our results showed that functional adenosine A_1 and A_2 receptors are present in newborn rat cardiomyocytes and that adenosine A_1 receptor activation by a specific agonist has a cytoprotective action on cells submitted to simulated ischemia-reperfusion, whereas adenosine A_2 receptor does not seem to be involved in cardiomyocyte protection.

2. Materials and methods

2.1. Cell culture

The primary cultures of neonatal rat cardiac myocytes were prepared from hearts of 2- to 4-day-old Wistar rat (Janvier, Le Genest St Isle, France) as previously described (Grynberg et al., 1986). Cardiomyocytes were obtained from ventricular tissue by seven dissociation steps of 10 min each at 33 °C in 0.1% trypsin (Difco, Detroit, MI, USA). The dissociated cells were collected by centrifugation and were suspended in standard culture medium composed of Ham's F-10 medium (Seromed, Berlin, Germany) supplemented with 20% fetal calf serum. To selectively increase the cardiac myocyte density, a two-step differential attachment technique was used (Grynberg et al., 1986). Finally, the cells were seeded in 60×15 Petri dishes (Falcon Primaria 3802, Becton Dickinson, Oxnard, CA, USA) at a final density of 2×10^6 cell/dish and incubated at 37 °C in a humidified atmosphere containing 5% CO_2, 19% O_2 and 79% N_2. The culture medium was changed 24 h after seeding and then every 2 days.

2.2. Contraction measurement

The contractions were monitored photometrically using a video motion detector. A universal counter (Harvard Apparatus, South Natick, MA, USA) continuously calculated the spontaneous rate of contractions. The contractile signals were displayed on the screen of a digital storage oscilloscope (Gould DSO 1604, Ilford, Essex, UK). These signals were stored on magnetic tape, using a frequency modulation tape recorder (Schlumberger MP 5521, Clermont-Ferrand, France), and transcribed onto paper chart with an ink jet oscillographic recorder (Siemens-Elema EM 81, Solna, Sweden). Measurements were done every 30 min of simulated ischemia (H1 to H5) or of reoxygenation (R1 to R3).

The contraction parameters are as follows: contraction rate, contraction duration at 80% full contraction (CD80), shortening time measured at 20% and 80% full contraction amplitude ($+C_{max}$), relaxation time measured at 20% and 80% full contraction amplitude ($-C_{max}$).

2.3. Procedures

Ischemic injury was simulated in vitro by using a substrate-free, hypoxia-reoxygenation model as previously described (Athias et al., 1987; Fantini et al., 1987). One hour before the experiments, the culture medium (5 ml) was replaced by glucose-free, Puck's F saline solution (in mM/l: NaCl 126.9, KCl 3.8, $CaCl_2$ 1.6, $MgSO_4$ 0.62, KH_2PO_4 0.61, $NaHCO_3$ 14.3, Na_2HPO_4 1.63) (Puck et al., 1958). This solution was then covered with a layer of paraffin oil. The cells were viewed with an inverted, phase-contrast microscope ($\times 510$, Diavert, Leica-Leitz, Wetzlar, Germany). The temperature was maintained at 36 ± 0.1 °C using a circulating heated water microchamber. During control normoxia and reoxygenation, the cell cultures were continuously flushed with air. Hypoxic conditions were achieved with a flow of nitrogen. The PO_2 values during normoxia, simulated ischemia and reoxygenation were 101.4 ± 2.1, 20 ± 6.3 and 89.3 ± 9.3 mm Hg, respectively.

2.4. Pharmacology

The stock solutions of adenosine, *R*-N^6-(2-phenylisopropyl)-adenosine (R-PIA) and 2-(4-[2-carboxyethyl]-

Table 1
Drugs used in the different protocols

Drugs	Abbreviations	Specificity
R-N^6-(2-phenylisopropyl)-adenosine	R-PIA	A_1 receptor agonist
1,3-dipropyl-8-cyclo-pentyl-adenosine	DPCPX	A_1 receptor antagonist
2-(4-[2-carboxyethyl]-phenethylamino)adenosine-5′*N*-ethylunosamide	CGS 21680	A_2 receptor agonist
3,7 dimethyl-1 propargylxanthine	DMPX	A_2 receptor antagonist

phen-ethyl-amino) adenosine-5′*N*-ethylunosamide (CGS 21680) were prepared in Puck G^- saline solution with 5% dimethylsulfoxide (DMSO, Sigma) (Table 1). Those of 3,7-dimethyl-1-propargylxanthine (DMPX) and 1,3-dipropyl-8-cyclo-pentyl-adenosine (DPCPX) were prepared with 70% ethanol. Stock solutions of each drug were prepared at 5.10^{-5} M and were added to the bath as 10-μl aliquots with a microsyringe (Hamilton, Bonaduz, Suisse) driven by a micromanipulator. The drugs were tested at the same concentration of 10^{-7} M. Homogeneous distribution was achieved by gentle mixing.

2.5. Protocols

The experimental protocols performed were the following: (i) short-term exposure, the contractile activity of cardiomyocytes placed in Puck G^- medium was recorded continuously during 1 h of normoxia and during 1 h after addition of the drugs to the bath medium; (ii) adenosine receptor inhibition by addition of DPCPX (adenosine A_1 receptor antagonist) or DMPX (adenosine A_2 receptor antagonist) 10 min before addition of the corresponding agonist; (iii) simulated ischemia, contractile activities being recorded during 1 h of control normoxia followed by 2.5 h of simulated ischemia and 1.5 h of reoxygenation; (iv) simulated preconditioning, the parameters being recorded during control normoxia (1 h) after addition of either R-PIA (adenosine A_1 receptor stimulation) or CGS 21680 (adenosine A_2 receptor stimulation) for 15 min followed by 2.5 h of simulated ischemia and 1.5 h of reoxygenation.

2.6. Statistical analysis

Three dishes were used in each protocol. In each dish, the recordings were made in three predetermined areas of the cell monolayer. The data were submitted to a two- or three-way analysis of variance, according to the protocols, with the culture effect as random factor and treatments (simulated ischemia-reoxygenation and/or drugs) as experimental fixed factors. Differences were considered significant at $P<0.05$.

3. Results

3.1. Determination of adenosine receptors

3.1.1. Effects of adenosine

The modifications of cardiomyocyte contractility induced by the addition of adenosine to the bath medium are presented in Table 2. Adenosine (10^{-7} M) induced a positive chronotropic response in cardiomyocytes. This effect was accompanied by a significant decrease in the shortening velocity ($-C_{max}$), which was reduced by 12%. The other changes in time parameters of contractions were moderate but not significant. The following experiments were thus designed to determine the adenosine receptors involved.

3.1.2. Adenosine A_1 receptors

The activation of adenosine A_1 receptors was achieved by using the selective agonist *R*-N^6-(2-phenylisopropyl)-adenosine (R-PIA). In some experiments, the preliminary blockade of adenosine A_1 receptors was achieved with 1,3-dipropyl-8-cyclo-pentyl-adenosine (DPCPX). The corresponding changes in contractile parameters induced by this adenosine A_1 receptor ligand are presented in Table 3. Addition of R-PIA induced a significant decrease in the frequency of spontaneous contractions (14%). This negative chronotropic effect was inhibited by DPCPX (Table 3), which had no functional influence when applied alone (data not shown). Otherwise, this chronotropic response of cardiomyocytes to R-PIA addition was not accompanied by significant changes in the parameters of contraction duration (CD20, CD80; Table 3) or in the rates of shortening ($+C_{max}$) and of relaxation ($+C_{max}$ and $-C_{max}$, respectively; Table 3).

3.1.3. Adenosine A_2 receptors

2-(4-[2-carboxyethyl]-phenethylamino) Adenosine-5′*N*-ethylunosamide (CGS 21680) was added to the bath as a selective agonist of adenosine A_2 receptors. The effects of the prior addition of 3,7 dimethyl-1 propargylxanthine

Table 2
Effects of adenosine on contractile parameters of rat cardiomyocytes in culture

	CR (min^{-1})	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Control	197.3 ± 13.9	46.5 ± 7.2	230.3 ± 16.3	63.0 ± 6.2	110.67 ± 17.8
Adenosine	220.4 ± 15.2	34.7 ± 11.3	227.3 ± 6.9	62.3 ± 7.4	97.3 ± 17.0
P	<0.05	NS	NS	NS	<0.05

CR: contraction rate; CD20 and CD80: contraction duration at 20% and 80% of relaxation, respectively; $+C_{max}$ and $-C_{max}$ shortening and relaxation velocities, respectively. Values represent means ± S.E.M. ($n=5$). The differences vs. control were considered significant at $P \leq 0.05$.

Table 3
Effects of adenosine A_1 receptor agonist (R-PIA, *R*-N^6-(2-phenylisopropyl)-adenosine) and its corresponding antagonist (DPCPX, 1,3-dipropyl-8-cyclo-pentyl-adenosine) on contractile parameters of rat cardiomyocytes in culture

	CR (min^{-1})	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Control	299 ± 51.7	52 ± 8.8	156 ± 24.4	41 ± 5.2	53 ± 8.5
R-PIA	256 ± 45.4	63 ± 10.9	167 ± 28.1	42 ± 5.6	60 ± 11.9
P	<0.05	NS	NS	NS	NS
Control	212 ± 44.9	59 ± 5.7	165 ± 6.8	42 ± 3.9	64 ± 7.3
DPCPX + R-PIA	213 ± 49.1	60 ± 5.7	168 ± 17.5	42 ± 3.4	65 ± 8.7
P	NS	NS	NS	NS	NS

CR: contraction rate; CD20 and CD80: contraction duration at 20% and 80% of relaxation, respectively; $+C_{max}$ and $-C_{max}$ shortening and relaxation velocities, respectively. Values represent means ± S.E.M. ($n = 5$). The differences vs. control were considered significant at $P \leq 0.05$.

(DMPX), an adenosine A_2 receptor antagonist, was assessed in parallel. The results obtained are presented in Table 4. In the presence of CGS 21680, cardiomyocytes displayed a significant increase in the spontaneous rate of contractions (+15%). This increase in rate was accompanied by a significant decrease in contraction duration at 20% and at 80% of relaxation (CD20, CD80; −13% and −17%, respectively; Table 4). Conversely, when supplied alone, the adenosine A_2 receptor agonist had no significant influence on the shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Pretreatment of cardiomyocytes with DMPX abolished the positive chronotropic action of CGS 21680. Similarly, the addition of DMPX suppressed the contraction shortening induced by the adenosine A_2 receptor agonist. Finally, the blockade of adenosine A_2 receptors did not influence the lack of effect of CGS 21680 on the shortening and relaxation times.

3.2. Effects of the simulated ischemia-reoxygenation

The absence of substrate in the experimental medium together with a flow of nitrogen (N_2) in the gas-controlled chamber for 2.5 h simulated in vitro the conditions of ischemia. Reoxygenation was achieved by restoration of the air flow in the chamber. After 1.5 h of reoxygenation, the PO_2 increased from 20 ± 6.3 mm Hg to 89.3 ± 9.3 mm Hg, a value not significantly different from that measured during control normoxia (101.4 ± 2.1 mm Hg). The changes in contractile parameters during normoxia (N), followed by 2.5 h of simulated ischemia (SI) (H1 to H5) and by 1.5 h of reoxygenation (R1 to R3), are illustrated in Fig. 1.

Simulated ischemia caused a progressive decrease and then arrest of the spontaneous contractile activity (Fig. 1A). Reoxygenation entailed a rapid resumption of the spontaneous contractions of cardiomyocytes, although the contraction rate remained below that measured during normoxia (Fig. 1A). The cardiomyocyte contractions ceased during simulated ischemia, and subsequent reoxygenation induced a complete recovery of this activity (Fig. 1B–E). During this period, the contraction duration (CD20 and CD80, respectively) and the shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively) were not significantly different from those measured during the control period (Fig. 1B–E, respectively).

3.3. A_1 pretreatment on simulated ischemia

3.3.1. Adenosine A_1 receptor stimulation

The in vitro model of preconditioning of rat cardiomyocytes in culture was achieved by addition of adenosine receptor ligands before the sequence of simulated ischemia-reoxygenation. The protocols were designed as follows (Figs. 2 and 3): (i) addition of the adenosine A_1 receptor agonist (R-PIA) 15 min before a 2.5-h episode of simulated ischemia-reoxygenation; (ii) addition of the adenosine A_1 receptor antagonist (DPCPX) and of the corresponding

Table 4
Effect of adenosine A_2 receptor agonist (CGS 21680, 2-(4-[2-carboxyethyl]-phen-ethyl-amino)adenosine-5′*N*-ethylunosamide) and its corresponding antagonist (DMPX, 3,7-dimethyl-1-propargylxanthine) on contractile parameters of rat cardiomyocytes in culture

	CR (min^{-1})	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Control	232 ± 31.1	60 ± 7.5	204 ± 25.3	58 ± 8.9	82 ± 16.1
CGS 21680	268 ± 33.2	44.3 ± 7.9	177 ± 22.9	54 ± 9.9	75 ± 10.9
P	<0.01	<0.01	<0.01	NS	NS
Control	210 ± 34.0	53 ± 9.8	187 ± 7.1	49 ± 4.1	64 ± 13.1
DMPX + CGS 21680	211 ± 38.9	54 ± 10.9	189 ± 11.3	48 ± 5.5	70 ± 15.7
P	NS	NS	NS	NS	NS

CR: contraction rate; CD20 and CD80: contraction duration at 20% and 80% of relaxation, respectively; $+C_{max}$ and $-C_{max}$ shortening and relaxation velocities, respectively. Values represent means ± S.E.M. ($n = 5$). The differences vs. control were considered significant at $P \leq 0.05$.

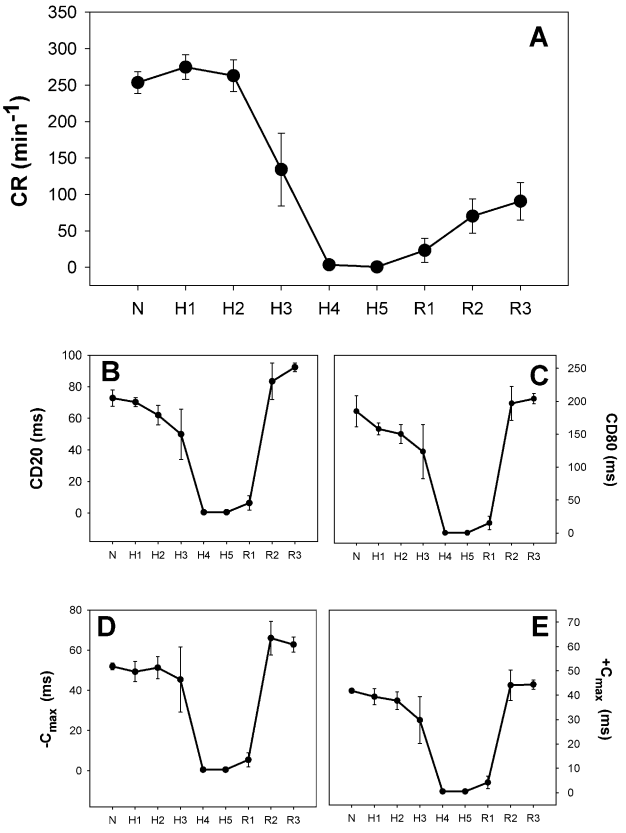

Fig. 1. Effects of simulated ischemia (SI) on cardiomyocyte functions during normoxia (N). H1, H2, H3, H4, H5: 0.5, 1, 1.5, 2 and 2.5 h of simulated ischemia, respectively; R1, R2, R3: 0.5, 1, 1.5 h of reoxygenation, respectively. (A) Contraction rate (CR); (B,C) contraction duration at 20% and 80% of relaxation (CD20 and CD80, respectively); (D,E) shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Values represent means ± S.E.M. ($n=3$). The differences vs. control (N) were considered significant at $P \leq 0.05$.

adenosine A_1 receptor agonist (R-PIA) before simulated ischemia-reoxygenation.

The activation of adenosine A_1 receptors by the addition of R-PIA induced a contractile response in cardiomyocytes similar to that previously described (Table 3), i.e., a negative chronotropic effect (Fig. 2A). In the presence of R-PIA, the decrease and the inhibition of spontaneous contractile activity induced by simulated ischemia were not observed. Indeed, the negative chronotropic effect observed during R-PIA pretreatment was followed by an increase in frequency at the beginning of simulated ischemia (125 to 214 min^{-1}). Then, after 1 h of simulated ischemia (H2), the spontaneous rate decreased gradually down to 28% of the initial value (164 min^{-1}) at the end of 2.5 h of simulated ischemia (H5). Furthermore, 30 min of reoxygenation was sufficient to restore the frequency, which was not significantly different from the control value (Fig. 2A). The other time parameters of contraction returned to values which did not significantly differ from control values (Fig. 2B–E). When DPCPX was applied before R-PIA, simulated ischemia induced a progressive decrease in the spontaneous rate (Fig. 3A) and in the other contractile parameters (Fig. 3B–E), which was similar to that obtained in the absence of drugs, although the cessation of contractile activity was more rapid than during control simulated ischemia (see H3, Fig. 1) in the absence of adenosine ligands. Moreover, in the presence of the combination of adenosine A_1 receptor agonist and antagonist, the reoxygenation failed to restore spontaneous contractile activity. This observation that contractile recovery was better in the absence of adenosine receptor blockade could be seen as being suggestive of a possible beneficial effect of a simulated ischemia-related release of endogenous adenosine. To summarize, adenosine A_1 receptor stimulation exerted a strong protective effect on cardiomyocytes against simulated ischemia, and this beneficial effect was inhibited by an adenosine A_1 receptor antagonist.

3.3.2. Adenosine A_2 receptor stimulation

To test whether adenosine A_2 receptor activation influenced the simulated ischemia-induced contractile failure, cardiomyocytes were submitted to the following protocols:

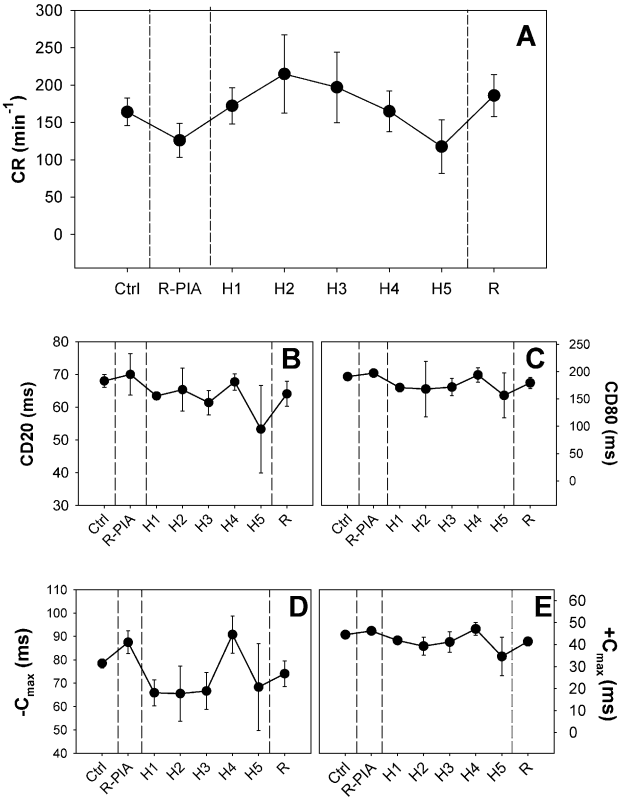

Fig. 2. Effects of adenosine A_1 agonist (R-PIA, R-N^6-(2-phenylisopropyl)-adenosine) pretreatment on the cardiomyocyte dysfunction induced by simulated ischemia (SI). Ctrl, control conditions (1 h). H1, H2, H3, H4, H5: 0.5, 1, 1.5, 2 and 2.5 h of simulated ischemia, respectively; R: 1.5 h of reoxygenation. (A) Contraction rate (CR); (B,C) contraction duration at 20% and 80% of relaxation (CD20 and CD80, respectively); (D,E) shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Values represent means ± S.E.M. ($n=3$). The differences vs. control (Ctrl) were considered significant at $P \leq 0.05$.

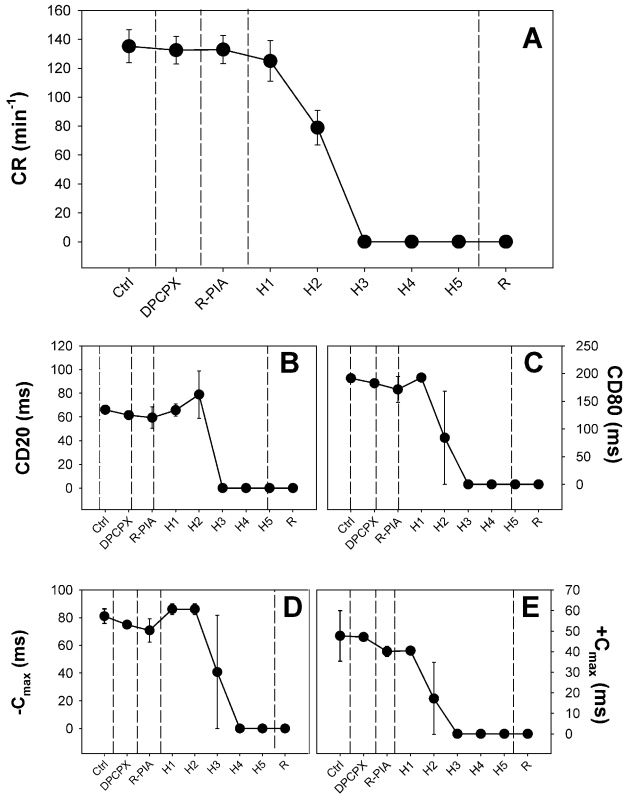

Fig. 3. Effects of adenosine A_1 antagonist (DPCPX, 1,3-dipropyl-8-cyclopentyl-adenosine) pretreatment followed by its corresponding agonist (R-PIA, R-N^6-(2-phenylisopropyl)-adenosine) on the cardiomyocyte dysfunction induced by simulated ischemia (SI). Ctrl, control conditions (1 h); DPCPX (10 min); R-PIA (15 min); H1, H2, H3, H4, H5: 0.5, 1, 1.5, 2 and 2.5 h of simulated ischemia, respectively; R: 1.5 h of reoxygenation. (A) Contraction rate (CR); (B,C) contraction duration at 20% and 80% of relaxation (CD20 and CD80, respectively); (D,E) shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Values represent means ± S.E.M. ($n=3$). The differences vs. control (Ctrl) were considered significant at $P \leq 0.05$.

(i) addition to the bath of adenosine A_2 receptor agonist (CGS 21680) 15 min before a 2.5-h episode of simulated ischemia-reoxygenation, and (ii) sequential addition of the adenosine A_2 receptor antagonist (DMPX) and of the corresponding adenosine A_2 receptor agonist (CGS 21680) before the simulated ischemia-reoxygenation sequence.

The effects of adenosine A_2 receptor activation via CGS 21680 on the consequences of simulated ischemia-reoxygenation are illustrated in Fig. 4. As already described above (see Table 4), the adenosine A_2 receptor agonist induced an increase in the spontaneous contraction rate (Fig. 4A). As observed with A_1 pretreatment, CGS 21680 also induced a further increase in the contraction rate during the first periods of simulated ischemia (H1–H2; Fig. 4A). Thereafter, this frequency parameter decreased until spontaneous contractile activity stopped. The contraction duration decreased significantly during the first hour of simulated ischemia (H2; Fig. 4B,C), and resumed transiently and slightly before contractions stopped (H5; Fig. 4B,C). The shortening and relaxation times ($+C_{max}$ and $-C_{max}$; Fig. 4D,E) decreased after CGS 21680 injection in normoxia and during the first hour of simulated ischemia (H2). During the second hour of simulated ischemia (H3–H4), a transient increase in these parameters was observed before the contractions stopped. After reoxygenation, cardiomyocytes contractile activity resumed, with the rate of contraction being not significantly different from that obtained during the control period. Conversely, the contraction duration parameters (CD20 and CD80; Fig. 4B,C) and the shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively; Fig. 4D,E) were slightly reduced.

The effects of simulated ischemia-reoxygenation after the successive addition of DMPX and then CGS 21680 are illustrated in Fig. 5. DMPX injection into the bath before the addition of CGS 21680 induced a decrease in the rate of contractions, while CGS 21680 reversed this change and caused the spontaneous rate to increase. During simulated ischemia, the frequency of the cardiomyocyte contractions decreased slightly during the first period of simulated ischemia after 1.5 h (H3; Fig. 5A). Afterwards, this diminution in

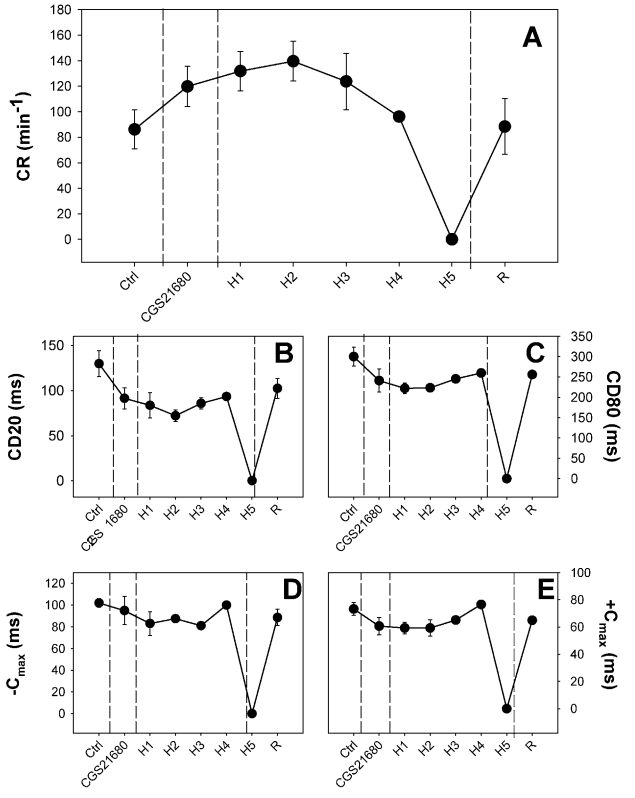

Fig. 4. Effects of adenosine A_2 agonist (CGS 21680, 2-(4-[2-carboxyethyl]-phen-ethyl-amino) adenosine-5′*N*-ethylunosamide) pretreatment on the cardiomyocyte dysfunction induced by simulated ischemia (SI). Ctrl, control conditions (1 h); CGS 21680 (15 min); H1, H2, H3, H4, H5: 0.5, 1, 1.5, 2 and 2.5 h of simulated ischemia, respectively; R: 1.5 h of reoxygenation. (A) Contraction rate (CR); (B,C) contraction duration at 20% and 80% of relaxation (CD20 and CD80, respectively); (D,E) shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Values represent means ± S.E.M. ($n=3$). The differences vs. control (Ctrl) were considered significant at $P \leq 0.05$.

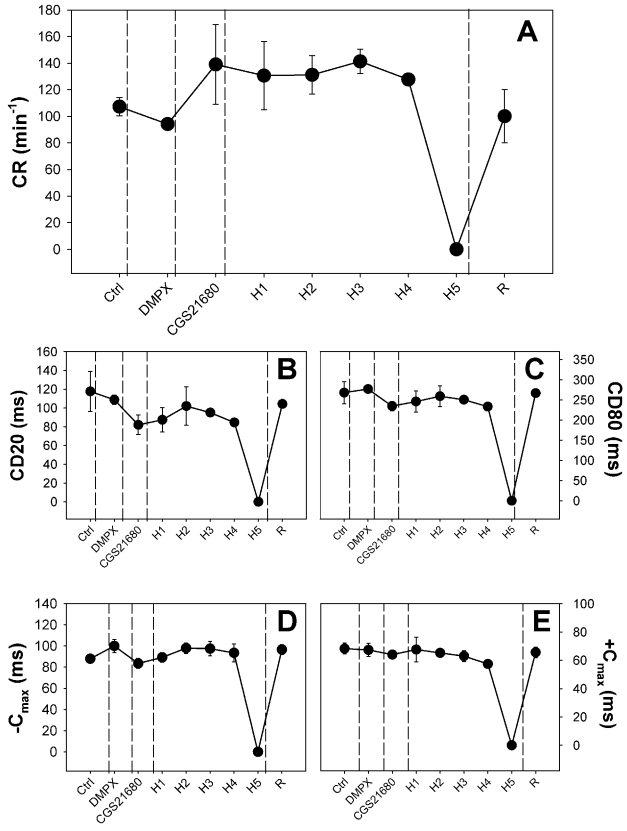

Fig. 5. Effects of adenosine A_2 agonist (DMPX, 3,7-dimethyl-1-propargylxanthine) pretreatment preceded by its corresponding antagonist (CGS 21680, 2-(4-[2-carboxyethyl]-phen-ethyl-amino) adenosine-5′*N*-ethylunosamide) on the cardiomyocyte dysfunction induced by simulated ischemia (SI). Ctrl, control conditions (1 h); DMPX (10 min); CGS 21680 (15 min); H1, H2, H3, H4, H5: 0.5, 1, 1.5, 2 and 2.5 h of simulated ischemia, respectively; R: 1.5 h of reoxygenation. (A) Contraction rate (CR); (B,C) contraction duration at 20% and 80% of relaxation (CD20 and CD80, respectively); (D,E) shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively). Values represent means ± S.E.M. ($n=3$). The differences vs. control (Ctrl) were considered significant at $P \leq 0.05$.

the spontaneous rate continued until cardiomyocyte contractile activity stopped (Fig. 5A). During simulated ischemia in the presence of both A_2 ligands, the contraction duration at 20% and 80% relaxation (CD20 and CD80, respectively; Fig. 4B,C) and the shortening and relaxation times ($+C_{max}$ and $-C_{max}$, respectively; Fig. 5D,E) progressively decreased over 2 h (H4) before contractile activity stopped (H5). During reoxygenation, the rhythmic cardiomyocyte contractions resumed and the values of the contraction parameters observed at the end of reoxygenation were close to those observed during the initial normoxic control period.

4. Discussion

Exposure of the heart to adenosine is known to decrease heart frequency and developed pressure. However, the influence of adenosine on the properties of the isolated ventricular myocyte and the intracellular mechanisms of action are less well known. The aim of the present work was to determine the subtypes of adenosine receptor in isolated rat ventricular myocytes and their putative role in protecting these cells against ischemia-reperfusion mimicked in vitro. Our results demonstrated that adenosine A_1 and A_2 receptors are present in isolated rat cardiomyocytes and that these cells can be protected against simulated ischemia-reperfusion by activation of adenosine A_1 receptors.

The determination of adenosine receptors in our model of newborn rat cardiomyocytes was done in several steps. First, we showed a positive chronotropic effect of the natural ligand adenosine at 10^{-7} M. This effect was in apparent contradiction with the action of adenosine on the mature myocardium under physiological conditions, in which negative chronotropic, dromotropic and inotropic negative responses mediated via adenosine A_1 receptors have been described (Schrader, 1990; Belardinelli, 1993; Shryock et al., 1997; Mubagwa et al., 1996). Nevertheless, this increase in contraction frequency is in accordance with that initially obtained by Seraydarian et al. (1972) with heart cell cultures. Also, Ponsard et al. (1999) observed a positive chronotropic effect of adenosine in the same experimental model, suggesting a peculiarity of newborn ventricular myocytes. This positive chronotropic effect of adenosine persisted after adenosine A_1 receptor antagonist (1,3-dipropyl-8-cyclo-pentyl-adenosine, DPCPX) addition before adenosine. Conversely, the presence of an adenosine A_2 receptor antagonist (3,7-dimethyl-1-propargylxanthine, DMPX) revealed a negative chronotropic effect of adenosine. These data suggested the coexistence in ventricular myocytes in culture of functional adenosine A_1 and A_2 receptors, whose stimulation gives rise to opposite effects on isolated cardiomyocyte spontaneous contractility.

In the myocardium, three adenosine receptors types have been detected, named A_1, A_{2A} and A_{2B} receptors (Müller and Stein, 1996). The addition of a selective adenosine A_1 receptor agonist (*R*-N^6-(2-phenylisopropyl)-adenosine; R-PIA) in our model of newborn rat cardiomyocytes induced a negative chronotropic effect, which was inhibited by the prior exposure of cardiomyocytes to DPCPX. The stimulation of cardiomyocytes by an adenosine A_2 receptor agonist (2-(4-[2-carboxyethyl]-phen-ethyl-amino)adenosine-5′*N*-ethylunosamide; CGS 21680) produced a positive effect which was specifically blocked by DMPX, the corresponding adenosine A_2 receptor antagonist. Therefore, newborn rat cardiomyocytes in culture possess both functional adenosine A_1 and A_2 receptors coupled with their respective effector systems, which are responsible for functional cell responses similar to those observed in vivo. As a result, the global functional response of these cells to the natural ligand is an average of the respective effects of adenosine A_1 and A_2 receptor agonists, which gave rise, in our model, to a predominantly positive A_2 effect. This is in agreement with the data of Shryock and Belardinelli (1997), suggesting that the ventricular myocardium content of adenosine A_2 recep-

tors exceeds that of adenosine A_1 receptors (Srinivas et al., 1997). More recent studies showed that the sensitivity of adenosine A_1 receptors in the heart decreased with age (Sawmiller et al., 1998; Gao et al., 1996). This age-dependant weakening of the A_1 response could be due to a decrease in the coupling efficiency between receptors and G_i proteins (Cai et al., 1997). Therefore, the cardioprotective potency of adenosine is assumed to vanish during development. Moreover, our results obtained with cultures of newborn myocardial tissue confirmed those obtained with intact preparations (Romano et al., 1989; Dobson and Fenton, 1997), but differed from previous reports suggesting maturation of these receptors with age. In rat ventricular myocytes, only the subtype A_{2A} was responsible for the cardiostimulatory effect of adenosine (Xu et al., 1996; Dobson and Fenton, 1997), whereas functional adenosine A_{2B} receptors have been demonstrated in a model of chick cardiomyocytes (Liang and Haltiwanger, 1995).

In the heart, the role of adenosine receptors in ischemic preconditioning has been widely studied (Yao et al., 1993; Pisarenko et al., 1997; Baxter et al., 1997). Adenosine receptors are also present in coronary vessels, neutrophils and platelets. Nevertheless, although their activation produces a beneficial effect against ischemia by increasing coronary flow (Giannella et al., 1997) and by decreasing leukocyte function, these vascular effects do not seem to contribute to preconditioning itself in the heart (Martin and Walter, 1996). Therefore, the beneficial effect of prior addition of adenosine may involve myocardial adenosine receptors. Since adenosine receptors have been detected in the cardiac myocytes in culture, the purpose of the work was also to study whether their activation could mimic preconditioning effects.

Rat cardiomyocytes in the substrate-free hypoxia model of simulated ischemia displayed contraction alterations comparable to those observed by Fantini et al. (1990) in the same experimental model. In brief, simulated ischemia caused a gradual decrease and then arrest of spontaneous contractile activity. During reoxygenation, cardiomyocyte contractile activity recovered and the contractile parameters reverted to values close to those observed under basal conditions. We have previously shown that these functional changes are accompanied by a decrease in ATP content, an increase in lactate production and the leakage of cellular lactate dehydrogenase (Chevalier et al., 1990). Reoxygenation restores the ATP content and attenuates the lactate loss. Moreover, these functional and biochemical alterations are similar to those observed during myocardial ischemia-reperfusion (Opie, 1991).

Our results show that adenosine A_1 receptor activation by R-PIA significantly reduced the contractile dysfunctions caused by simulated ischemia. Indeed, in the presence of the adenosine A_1 receptor agonist, spontaneous contractile activity was maintained, even after 2.5 h of simulated ischemia, and reoxygenation induced a fast recovery of contractile parameters. The cardioprotective role of adenosine A_1 receptors has also been demonstrated in vivo and in vitro, although species differences have been observed. Neely et al. (1996) initially considered that the beneficial effects of adenosine A_1 receptor pretreatment with a specific agonist were due to inhibition of A_1 signalling mechanisms. However, these earlier results were contradicted by several studies showing that the cardioprotective effects of adenosine are directly related to the intracellular signalling resulting from the activation of its receptors (Shryock et al., 1997). These data were also in agreement with the present results showing that adenosine A_1 receptor inhibition by DPCPX before simulated ischemia completely inhibited the beneficial effects obtained with R-PIA. Our results are thus consistent with the view that the stimulation of adenosine A_1 receptors mimics the ischemic preconditioning effects described in vivo and that the cardiomyocyte itself is a target of this phenomenon. The type of myocardial adenosine receptors involved in this cardioprotective phenomenon seems to be species dependent. Liang (1996) showed that the chick embryo ventricular myocytes were also protected against the noxious effects of hypoxia by a prior exposure to adenosine A_1 receptor agonist, although a contributory role of adenosine A_3 receptors could not be excluded in the preconditioning effects. Also, it seems that preconditioning in the intact myocardium as well as isolated cardiomyocytes required adenosine A_1 and A_3 receptor activation (Wang et al., 1997; Giannella et al., 1997). In the rabbit, the degree of protection obtained through adenosine A_3 receptors was similar to that obtained through adenosine A_1 receptors (Tracey et al., 1997). Overexpression of adenosine A_1 and A_3 receptors in chick embryo cardiac cells seems to protect cells against ischemia-reperfusion (Dougherty et al., 1998). In the isolated rat heart, Hu and Nattel (1995) showed that R-PIA prolonged the delay of ischemic spasms, interpreting this effect as a reduction in energy demands during ischemia. Pisarenko et al. (1997) showed in the dog that R-PIA pretreatment did not affect cardiac hemodynamic parameters but induced a significant reduction in lactate, creatine and inorganic phosphate release during ischemia, as well as a better recovery of ATP content and of phosphocreatine during reperfusion.

Conversely, the results obtained in the present work show that activation of cardiomyocyte adenosine A_2 receptors by the selective agonist (CGS 21680) failed to alleviate the noxious effects of simulated ischemia. Indeed, reoxygenation did not produce a complete recovery of contraction duration and time parameters. Moreover, when adenosine A_2 receptor agonist was preceded by its corresponding antagonist (DMPX), the functional changes induced by simulated ischemia-reoxygenation were similar to those noted in the absence of A_2 ligands, with a near complete recovery of contractile activity during reoxygenation. The activation of adenosine A_{2A} receptors in cardiomyocytes attenuated the effects of adenosine in preconditioning (Strickler et al., 1996). In the isolated rat heart, adenosine A_{2A} receptors may also be involved in the protection of the myocardium against ischemia-reperfusion, but by mecha-

nisms different from those of adenosine A_1 receptors (Lozza et al., 1997).

The activation of protein kinase C (PKC) may be involved in this cardioprotective action. Indeed, PKC inhibition by calphostine C abolished ischemic adenosine-induced preconditioning in rabbit cardiomyocytes (Armstrong and Ganote, 1995). PKC seems to play an important role in the protection of newborn rat cardiomyocytes against the cellular death induced by hypoxia (Gray et al., 1997). Also, phorbol ester activator of PKC in cardiomyocytes in culture mimicked the effects of ischemic or adenosine preconditioning (Ikonomidis et al., 1994). These authors suggested that adenosine release from myocytes during ischemia was promoted by PKC activation. Otherwise, the cardioprotective action of adenosine has been assumed to be related to potassium channels (K_{ATP}). The activation of these channels by pinacidil decreased chick cardiomyocyte death in culture (Liang, 1996). The stimulation of adenosine A_1 receptors may also reduce cardiomyocyte injury by opening K_{ATP} channels (Narayan et al., 2001). Moreover, glibenclamide, a K_{ATP} inhibitor, abolished preconditioning induced by adenosine in the isolated rat heart and in the rabbit (Yao et al., 1993; Kouchi et al., 1998; Toombs et al., 1993).

In summary, the specific activation of adenosine A_1 receptors in cardiomyocytes protects cardiac muscle cells against damage induced by simulated ischemia, and reoxygenation induced a complete recovery of the contractile parameters. However, when the corresponding antagonist (DPCPX) was applied before R-PIA, cardiomyocyte contractile activity abolished during simulated ischemia was not restored during reoxygenation, which additionally suggests the discrete release of adenosine in our in vitro cellular model of ischemia-reoxygenation. Under hypoxic conditions, this endogenous production may exert a protection effective enough to favor cardiomyocyte recovery during reoxygenation. Adenosine A_2 receptor activation by CGS 21680 did not attenuate the simulated ischemia-induced depression of cardiomyocyte contractility. Furthermore, during reoxygenation, none of the contractile parameters, except frequency, reverted to values close to those observed in the control period. Moreover, the inhibition of adenosine A_2 receptor activation by the corresponding antagonist induced a complete recovery of the contractile parameters after simulated ischemia-reperfusion, leading to cardiomyocyte function similar to that obtained during simulated ischemia-reoxygenation in the absence of the drugs.

In conclusion, our work shows for the first time that rat cardiomyocyte contractile function in culture can be protected from the deleterious effects of simulated ischemia by a pharmacological treatment intended to mimick adenosine-dependant ischemic preconditioning effects. This study tends to confirm the dominant involvement of adenosine A_1 receptors in this cardioprotective process.

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