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Predominant role of A_1 adenosine receptors in mediating adenosine induced vasodilatation of rat diaphragmatic arterioles: involvement of nitric oxide and the ATP-dependent K^+ channels

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1 We investigated, by intravital microscopy in rats, the role of the subtypes of adenosine receptors A_1 (A_1/AR) and A_2 (A_2AR) in mediating adenosine-induced vasodilatation of second and third order arterioles of the diaphragm.

2 Adenosine, and the A_1AR selective agonists **R**(−)-N^6-(2-phenylisopropyl)-adenosine (**R**-PIA) and N^6-cyclo-pentyl-adenosine (CPA) induced a similar concentration-dependent dilatation of diaphragmatic arterioles. The non selective A_2AR subtype agonist N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl) ethyl]adenosine (DPMA) also dilated diaphragmatic arterioles but induced a significantly smaller dilatation than adenosine. By contrast the selective A_{2a}AR subtype agonist 2-[p-(2-carboxyethyl)phenyl amino]-5′-N-ethyl carboxamido adenosine (CGS 21680) did not modify diaphragmatic arteriolar diameter.

3 The non selective adenosine receptor antagonist 1,3-dipropyl-8-*p*-sulphophenylxanthine (SPX, 100 μM) and the selective A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX, 50 nM) significantly attenuated adenosine-induced dilatation of diaphragmatic arterioles. By contrast, adenosine significantly dilated diaphragmatic arterioles in the presence of A_2AR antagonist 3,7-dimethyl-1-propargylxanthine (DMPX, 10 μM).

4 The dilatation induced by adenosine was unchanged by the mast cell stabilizing agent sodium cromoglycate (cromolyn, 10 μM).

5 The nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine (L-NOARG, 300 μM) attenuated the dilatation induced by adenosine, and by the A_1AR and A_2AR agonists.

6 The ATP-dependent K^+ channel blocker glibenclamide (3 μM) significantly attenuated diaphragmatic arteriolar dilatation induced by adenosine and by the A_1AR agonists **R**-PIA and CPA. By contrast, glibenclamide did not significantly modify arteriolar dilatation induced by the A_2AR agonist DPMA.

7 These findings suggest that adenosine-induced dilatation of diaphragmatic arterioles in the rat is predominantly mediated by the A_1AR, via the release of NO and activation of the ATP-dependent K^+ channels.

Keywords: Diaphragm; arterioles; adenosine receptors; **R**-PIA; DPMA; DPCPX; DMPX; DPSPX; nitric oxide; glibenclamide

Introduction

The work of the respiratory muscles, especially the diaphragm, is required for maintaining adequate pulmonary ventilation. The performance of these muscles is tightly coupled to blood flow. For example, recent studies have indicated that mechanical hyperperfusion of the diaphragm partially delays the development of diaphragmatic fatigue (Ward *et al*., 1992) and reverses the loss of force generating capacity induced by repetitive diaphragmatic contractions (Supinski *et al*., 1988). Blood flow to the diaphragm is highly dependent on changes in muscle metabolic needs (Hussain *et al*., 1989) and hypoxaemia (Bark *et al*., 1988). Regulation of diaphragmatic blood flow involves metabolic, myogenic and endothelial influences on the tone of diaphragmatic microvessels. Whereas the role of endothelium derived substances such as nitric oxide has been described in detail (see Hussain, (1996) for review), no data are available in the current literature concerning the role and the mechanism(s) of action of adenosine, which is a main metabolic determinant of vascular tone (Berne, 1963; Gustafsson *et al*., 1990). Indeed, adenosine is a potent vasodilator that is thought to be an important contributor to the metabolic feedback mechanisms that affect local control of blood flow. Furthermore, adenosine has been postulated to be a major mediator of vascular adaptation to hypoxia (Marshall *et al*., 1993). It is, therefore, relevant to assess the effect and the mechanism(s) underlying the actions of adenosine on diaphragmatic microcirculation.

It is generally accepted that adenosine-induced vasodilatation is primarily mediated through the A_2 receptor (A_2AR) as classified pharmacologically according to the relative rank order of potency of a number of adenosine analogues (Kusachi *et al*., 1983; Mustafa & Askar, 1985; McCormack *et al*., 1989; Stojanov & Proctor, 1989; Gustafsson *et al*., 1990; Merkel *et al*., 1992; Ngai & Winn, 1993; Haynes *et al*., 1995). However, in the coronary arteries it has been suggested that the A_1 receptor (A_1AR) may co-participate with the A_2AR in mediating vasodilatation (Merkel *et al*., 1992). Conclusive data regarding the mechanism(s) whereby adenosine receptors mediate vasodilatation are still limited. Adenosine can interact with parenchymal cells surrounding arterioles such as the mast cells (Doyle *et al*., 1994). As a result of this interaction, mast cells can release vasoactive substances which in turn can modulate the effects of adenosine on vessel diameter. Adenosine can also interact with endothelial cells, promoting the release by these cells of the endogenous vasodilator nitric oxide (NO) (Li *et al*., 1995). Finally, another factor that might contribute to the vascular effects of adenosine is the adenosine 5′-triphosphate (ATP)-dependent K^+ channel, because adenosine has been shown to activate this channel in vascular smooth muscle cells (Nelson *et al*., 1990; Dart & Standen, 1993).

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The aim of this study was, therefore, to evaluate the effects of adenosine on diaphragmatic microcirculation. We specifically investigated, by using pharmacological tools and intravital microscopy, the role of the A_1AR and the A_2AR in mediating adenosine-induced vasodilatation of rat diaphragmatic arterioles. Because two different subtypes of the A_2AR have been identified (A_{2a} and A_{2b}, respectively) we investigated their respective role in the effects of adenosine. We also studied the mechanism(s) whereby adenosine receptors mediated diaphragmatic arteriolar dilatation. For this purpose, we examined the role of products of mast cell degranulation and the contributions of NO and ATP-dependent K^+ channels.

Methods

Animals

One hundred and sixty-eight male albino rats (159 ± 10 g) of the Sprague-Dawley strain were obtained from Charles River France Inc. All rats were housed individually, acclimatized to a 12 h light dark cycle, and maintained on Purina rat chow and tap water *ad libitum* for a 5 day period before being used for experiments.

The animals were anaesthetized with an intraperitoneal injection of 50 mg kg^{-1} sodium pentobarbitone and placed in a supine position on a rodent operating table (Harvard Apparatus, MA). After tracheotomy, the animals were mechanically ventilated (FIO_2 of 50%) with a rodent ventilator (Ugo Basile Apparatus, Italy). The left carotid artery was cannulated for continuous measurement of systemic arterial blood pressure with a Statham P23XL transducer (Spectramed Ltd., Coventry, U.K.). A second catheter was placed into the right jugular vein to administer 5 ml kg^{-1} of sterile physiological solution (NaCl 0.9%) in order to compensate for liquid losses during the surgical procedure. Rectal temperature was continuously monitored with a thermistor and maintained constant at 37°C by a heat lamp and a heating pad (Harvard Apparatus, MA).

Preparation of the diaphragm

The diaphragm preparation has been previously described in detail (Boczkowski *et al*., 1990).

Briefly, a bilateral thoracotomy avoiding the sternum was performed in the fifth intercostal space. The diaphragm was carefully separated from the lungs and from the mediastinal tissues. Then, the abdomen was opened by a midline laparotomy which was followed by a transversal incision in order to expose and to place the diaphragm in a perpendicular position relative to the body of the animal. The abdominal organs were covered with cotton sheets moistened with warm saline solution. The animal was placed in the Trendelenburg position.

Irrigation of the abdominal side of the diaphragm began immediately after exposure with a modified Krebs-Henseleit solution containing (in mM): NaCl 118, KCl 5.9, $CaCl_2.2H_{20}$ 2.5, $MgSO_4.7H_20$ 0.5, $NaHCO_3$ 26 and glucose 10. This solution was maintained at a constant temperature of 37.5°C. By bubbling the solution with a 6% $C0_2$-94% N_2 gas mixture, the pH, PO_2, and PCO_2 of this solution were fixed at 7.41 ± 0.06, 22 ± 1.6 and 41 ± 0.4 T, respectively. Pancuronium bromide, 40 μM (Pavulon), was added to this solution to prevent muscle fasciculation. This dose of pancuronium had no effect on microcirculatory parameters (Faber & Harris, 1981).

The muscle was transilluminated with a fibreoptic cool light microprobe gently introduced into the thorax via the left thoracotomy. The costal diaphragmatic microcirculation was visualized by observing the abdominal side of the muscle with a movable optic microscope (Leitz Inc., Germany) whose objective was placed in a position parallel to the area of the muscle under observation. The image was magnified by a $20 \times$ long-distance objective, and projected into a CCD video camera (Sony DXC-101P) connected to a videotape recorder (Sony VO-9600 P) and a video monitor (Sony PVM-1371 QM).

Microvascular anatomy

Changes in diameter of the second and third order diaphragmatic arterioles were measured. The arteriolar network was observed to begin at the point where the first order arteriole, which arose from the internal mammary or intercostal arteries, entered the muscle via its costal margin. It exhibited three to four successive bifurcations, each one corresponding to a successive arteriolar order (Zweifach & Lipowski, 1984). Diameter of second and third order arterioles was about 40 and 25 μm, respectively. Depending on the geometry of the part of the network studied, the changes in diameter of one to three arterioles in each animal were determined.

Arteriolar diameter was measured by selecting a clearly distinguishable arteriolar network. Care was taken to visualize the midplane of the vessel by bringing into focus its sharpest outer borders and widest image. Arteriolar diameters were measured by playback analysis of the video record, by use of the technique of Intaglietta and Tompkins (1973) and a distance measurement device (IPM 303 Dimension Analyser, San Diego).

Experimental protocol

Six sets of experiments were performed. Each set of experiments included one or several different groups of animals. In the following paragraphs *N* refers to the number of animals, and *n* to the number of arterioles studied in each group.

The general protocol of the experiments was the following: after surgery was completed, a 20–30 min period was allowed for the arteriolar tone to reach a steady state before baseline diameters were measured. Then, a cumulative concentration-response curve of diaphragmatic arteriolar diameter to an agonist was performed, by diluting the drug in the Krebs-Henseleit solution and by superfusing it in a stepwise fashion in a range of concentrations varying from 10 nM to 100 μM. Individual concentrations were given until the arteriolar diameter remained stable for at least 2 min and then the diameter of the selected arteriole was measured. Even at the lowest drug concentrations, steady state diameters of the arterioles were achieved within 1 min.

Effect of adenosine and A_1 and A_2 receptor agonists This series of experiments was carried out to compare diaphragmatic arteriolar dilatation induced by adenosine and by the specific A_1AR agonists **R**-N^6-phenylisopropyladenosine (**R**-PIA) (Daly *et al*., 1981) and N^6-cyclo-pentyl-adenosine (CPA) (Moos *et al*., 1985) and by the specific A_2AR agonists N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine (DPMA) (Bridges *et al*., 1988) and 2-[p-(2-carboxyethyl)phenyl amino]-5′-N-ethyl carboxamido adenosine (CGS 21680; Hutchinson *et al*., 1989; Jarvis *et al*., 1989). Note that DPMA is a non-selective A_2AR subtype agonist and CGS 21680 is selective for the A_{2a}AR subtype (Jarvis *et al*., 1989).

After measurement of baseline arteriolar diameters, the animals were allocated to 5 distinct experimental groups in which concentration-response curves either to adenosine, or to one of the selective agonists **R**-PIA, CPA, DPMA or CGS 21680 were obtained.

Selectivity of adenosine receptor (A_1 vs A_2) antagonists This series of experiments was carried out to evaluate the selectivity of 1,3-dipropyl-8-cyclopentylxanthine (CPX), considered to be a specific antagonist of the A_1AR (Daly *et al*., 1985; Bruns *et al*., 1987; Peet *et al*., 1990), and of 3,7-dimethyl-1-propargylxanthine (DMPX), considered to be a specific antagonist of the A_2AR (Ukena *et al*., 1986).

After baseline arteriolar diameters had been measured, the animals were allocated to 4 distinct experimental groups in which concentration-response curves to **R**-PIA or to DPMA in the absence and presence of 50 nM CPX or 10 μM DMPX were obtained.

Effect of adenosine receptor antagonists on adenosine-induced diaphragmatic arteriolar dilatation This series of experiments was carried out to evaluate the effect of adenosine-receptor subtype antagonists on adenosine-induced diaphragmatic arteriolar dilatation. After baseline arteriolar diameters had been measured, the animals were allocated to 3 distinct experimental groups in which concentration-response curves to adenosine in the presence of either 100 μM 1,3-dipropyl-8-sulphophenylxanthine (SPX), a non-selective adenosine receptor antagonist (Daly *et al*., 1985; Peet *et al*., 1990; $N=10$), CPX ($N=8$) or DMPX ($N=10$) were obtained.

Role of mast cells degranulation products on adenosine-induced diaphragmatic arteriolar dilatation This series of experiments was carried out to determine the effect of the mast cell stabilizing agent sodium cromoglycate (cromolyn) on arteriolar dilatation induced by adenosine. After measurement of baseline arteriolar diameters, a concentration-response curve to adenosine was performed in the presence of 10 μM of cromolyn ($N=6$ animals). This concentration of cromolyn has been previously shown to inhibit degranulation of periarteriolar mast cells caused by adenosine (Doyle *et al*., 1994).

Role of NO on diaphragmatic arteriolar dilatation induced by adenosine and by adenosine analogues This series of experiments was carried out to determine the effect of N^{ω}-nitro-L-arginine (L-NOARG), a very potent and specific inhibitor of NO synthesis *in vitro* (Gross *et al*., 1990; Buga & Ignarro, 1992; Hecker *et al*., 1990) and *in vivo* (Mügge *et al*., 1991; Benyo *et al*., 1992; Hussain *et al*., 1992), on arteriolar dilatation induced by adenosine and by adenosine analogues, except CGS 21680 which did not dilate diaphragmatic arterioles. After baseline diameter of the selected arterioles had been measured, the diaphragm was superfused with Krebs solution containing L-NOARG (300 μM). After a 20 min period, the diameter of the selected arterioles was measured, and the animals were allocated to 4 distinct experimental groups in which concentration-response curves to adenosine, **R**-PIA, CPA and DPMA ($N=6$ in each group) were obtained. Superfusion of L-NOARG was maintained throughout the whole experiment.

In a previous study we demonstrated that L-NOARG, at the concentration used in the present experiments (300 μM), selectively inhibits synthesis of NO in diaphragmatic arterioles (Boczkowski *et al*., 1994).

Role of ATP-dependent K^+-channel on diaphragmatic arteriolar dilatation induced by adenosine and by adenosine analogues This series of experiments was carried out to evaluate the effect of glibenclamide, an ATP-dependent K^+ channel blocker (Standen *et al*., 1989), on arteriolar dilatation induced by adenosine and by adenosine analogues, except CGS 21680 which did not dilate diaphragmatic arterioles. After baseline diameter of the selected arterioles had been measured, the diaphragm was superfused with Krebs-Henseleit solution containing glibenclamide (3 μM). After a 20 min period, the diameter of the selected arterioles was measured, and the animals were allocated to 4 distinct experimental groups in which concentration-response curves to adenosine, **R**-PIA, CPA, and DPMA ($N=11$, 12, 6 and 6, respectively) were obtained. Superfusion of glibenclamide was maintained throughout the experiment.

In previous experiments ($N=9$), we determined that glibenclamide (3 μM) significantly blocked second and third order diaphragmatic arteriolar dilatation induced by cromakalin, an ATP-dependent K^+ channel opener (Standen *et al*., 1989).

Drugs

Pancuronium bromide, adenosine, cromolyn, glibenclamide and L-NOARG were obtained from Sigma Chemical Co (St. Louis, MO). **R**-PIA, CPA, DPMA, CGS 21680, SPX, CPX and DMPX were obtained from Research Biomedicals Inc. (Natick, Mass). Adenosine, sodium cromoglycate, glibenclamide and L-NOARG were directly dissolved in the Krebs-Henseleit solution. **R**-PIA, CPA, DPMA, CGS 21680, (2-[p-(2-carboxyethyl)phenyl amino]-5′-N-ethyl carboxamide adenosine), SPX, CPX and DMPX were made up at a concentration of 100 μM in 0.2% dimethylsulphoxide (DMSO, Sigma Chemical Co, St. Louis, MO). Further dilutions of these drugs were made in the Krebs-Henseleit solution as required. Final DMSO concentration in the bath did not exceed 0.2%. This concentration of DMSO had no effect on the responses of diaphragmatic arterioles to adenosine receptor agonists and antagonists. Drug solutions were prepared fresh daily.

Data analysis

Data are presented as means ± s.e.mean. Comparison between arteriolar diameters at baseline and after superfusion of inhibitors in the different sets and groups of experiments

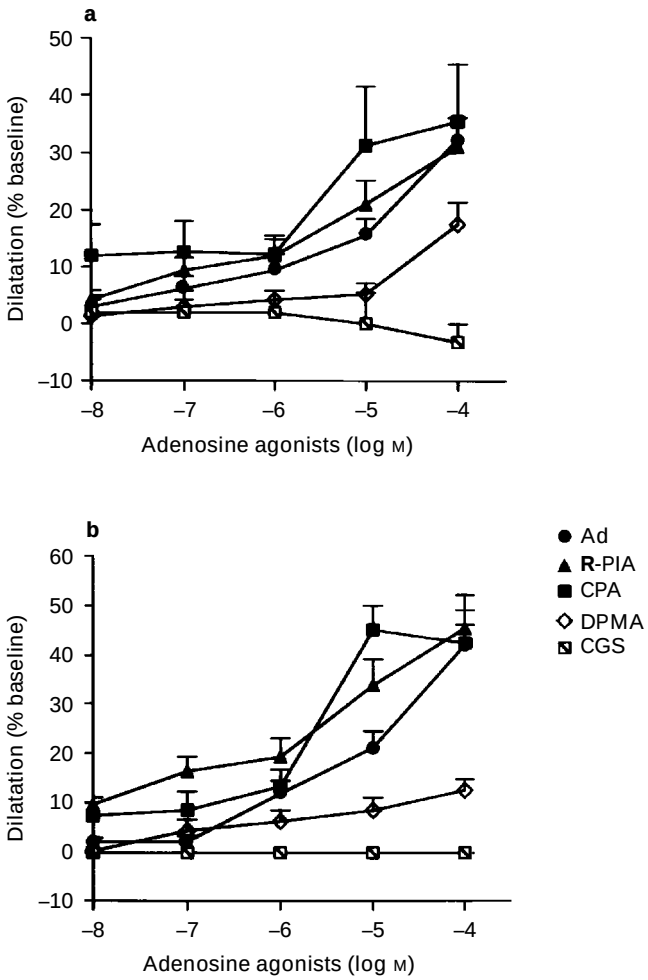

Figure 1 Concentration-response curves of (a) second and (b) third order diaphragmatic arterioles to adenosine (Ad), to the A_1 selective agonists **R**-PIA and CPA, to the A_2 selective agonist DPMA and the A_{2a} subtype selective agonist CGS 21680 (CGS). Data are expressed as mean and vertical lines show s.e.mean; $N=6$ to 8 animals, and $n=10$ to 25 arterioles per group respectively. Note that whereas **R**-PIA and CPA mimicked the effect of adenosine, DPMA induced a dilatation significantly smaller than adenosine, and CGS 21680 did not significantly modify arteriolar diameter.

was performed by one way analysis of variance. Comparisons of the effects of adenosine and adenosine receptor agonists on arteriolar diameter in the presence or absence of the different antagonists and inhibitors, was performed by comparing the concentration-response curves by use of two-way analysis of variance for repeated measurements (Winer, 1971) considering one 'grouping' factor (i.e. factor group) and one 'within' factor (i.e. factor concentration). Two by two comparisons between the concentration-response curves were made only when the overall comparison was significant. In some experiments, an abrupt increase in the maximal response of the concentration-response curve was observed. In these cases, a comparison between maximal responses was performed by one way analysis of variance. Differences between means were considered statistically significant when $P<0.05$.

Results

The experimental interventions did not affect systemic blood pressure (95–120 mmHg), which was stable during the entire course of the experiments. Baseline arteriolar diameters within various experimental groups ranged from 36.02 ± 1.16 to 41.87 ± 3.96 μm and 24.85 ± 1.24 to 27.76 ± 1.85 μm for second and third order arterioles, respectively, and were not significantly different between the different experimental protocols.

Effect of adenosine and A_1 and A_2 receptor agonists

As shown in Figure 1, adenosine, **R**-PIA, CPA and DPMA induced a statistically significant concentration-dependent dilatation of second order diaphragmatic arterioles. It should be noted that the responses to these agents did not reach maximal values. Concentrations of agonists higher than 10^{-4} M induced arterial hypotension. For this reason, the highest concentration used of these agonists was 10^{-4} M.

Analysis of the concentration-response curves of second order arterioles to the A_1AR agonists, **R**-PIA and CPA, revealed that they were not significantly different. A trend towards a greater dilatation with these agonists than with adenosine was observed. However, this difference did not reach statistical significance ($P<0.05$).

By contrast, dilatations of second order arterioles induced by the non-selective A_2AR subtype agonist DPMA were significantly smaller than the dilatations caused by adenosine, **R**-PIA and CPA. It should be noted that the shape of the concentration-curve for DPMA changed abruptly at the highest concentration (10^{-4} M); the increase in diameter observed with this concentration amounted to 17% of baseline as compared to 4 and 5% for 10^{-6} M and 10^{-5} M, respectively. In contrast to DPMA, the A_{2a}AR subtype selective agonist CGS 21680 caused no change in arteriolar diameter.

The responses for third order arterioles were similar to that observed in second order vessels. There was a trend towards a higher responsiveness of third order arterioles to adenosine, **R**-PIA and CPA, but less to DPMA. However, these differences did not reach statistical significance ($P>0.05$).

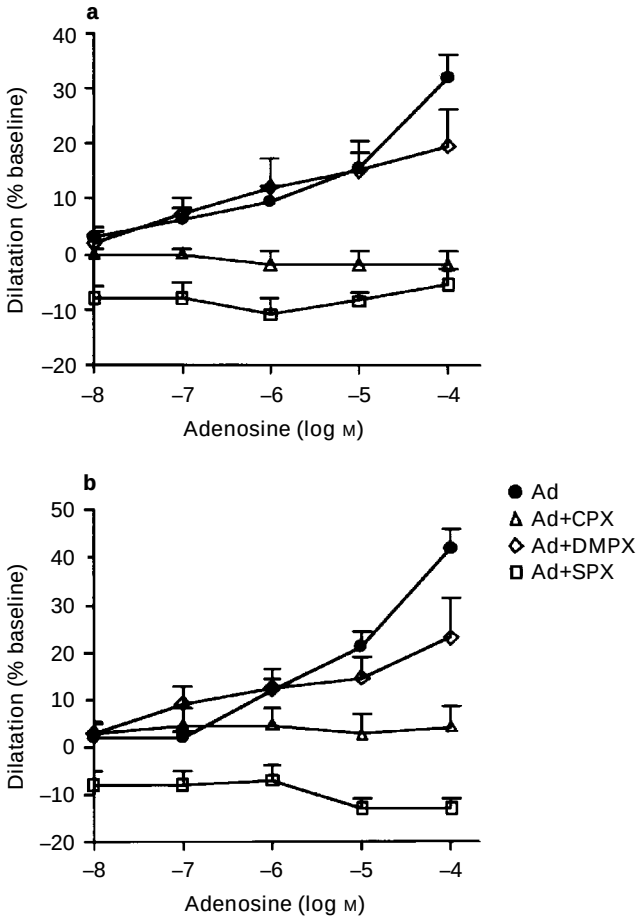

Figure 2 Concentration-response curves of (a) second and (b) third order diaphragmatic arterioles to adenosine (Ad) in the absence and presence of CPX (A_1AR selective antagonists), DMPX (A_2AR selective antagonist), or SPX (non-selective antagonist). Data are expressed as mean and vertical lines show s.e.mean; $N=8$ to 10 animals and $n=8$ to 24 arterioles per group, respectively. Note that only CPX and SPX significantly inhibited adenosine-induced vasodilatation of both order diaphragmatic arterioles.

Table 1 Diaphragmatic arteriolar responses to **R**-PIA and to DPMA in the presence of CPX or DMPX

Second order arterioles, dilatation (% baseline)

	In the absence of antagonist			*In the presence of CPX (5×10^{-8} M)*			*In the presence of DMPX (10^{-5} M)*		
	10^{-8} M	10^{-6} M	10^{-4} M	10^{-8} M	10^{-6} M	10^{-4} M	10^{-8} M	10^{-6} M	10^{-4} M
R-PIA	4.4 ± 1.5	12.1 ± 2.9	31.2 ± 5.1	0.1 ± 0.1*	-6.4 ± 4.1*	-7.4 ± 3.5*	4.3 ± 1.3	15.2 ± 2.8	33.2 ± 5.5
DPMA	1.3 ± 0.1	4.3 ± 1.6	17.4 ± 3.9	1.2 ± 0.2	5.2 ± 1.5	18.5 ± 3.5	0.2 ± 0.1	0.1 ± 0.1*	0.2 ± 0.2*

Third order arterioles, dilatation (% baseline)

	In the absence of antagonist			*In the presence of CPX (5×10^{-8} M)*			*In the presence of DMPX (10^{-5} M)*		
	10^{-8} M	10^{-6} M	10^{-4} M	10^{-8} M	10^{-6} M	10^{-4} M	10^{-8} M	10^{-6} M	10^{-4} M
R-PIA	9.5 ± 1.7	19.14 ± 3.7	45.6 ± 6.7	0.2 ± 0.1*	-5.3 ± 2.1*	-6.1 ± 1.9*	9.2 ± 1.7	13.2 ± 3.7	42.1 ± 6.1
DPMA	0.4 ± 0.1	6.04 ± 2.4	12.4 ± 2.1	0.1 ± 0.1	5.5 ± 2.4	14.2 ± 2.1	0.2 ± 0.2	0.2 ± 0.1*	0.1 ± 0.1*

Data are expressed as mean$\pm$s.e.mean; $N=6$ animals and $n=4$ to 25 arterioles per group respectively. *$P<0.001$ as compared to the same agonist in the absence of antagonist.

Selectivity of adenosine receptor antagonists

Table 1 shows diaphragmatic arteriolar responses to **R**-PIA and to DPMA in the absence and presence of CPX or DMPX. In both second and third order vessels the dilatation induced by **R**-PIA was significantly reduced in the presence of CPX whereas it was not significantly modified in the presence of DMPX. By contrast, the dilatation induced by DPMA was significantly reduced in the presence of DMPX whereas it was not significantly modified in the presence of CPX.

Effect of adenosine receptor antagonists on adenosine-induced diaphragmatic arteriolar dilatation

Figure 2 shows the effects of the A_1AR antagonist CPX, the A_2AR antagonist DMPX and the non specific adenosine receptor antagonist SPX on diaphragmatic arteriolar dilatation induced by adenosine.

In the presence of SPX or CPX, adenosine induced no dilatation of second and third order diaphragmatic arterioles. Furthermore, it should be noted that a small, but significant, arteriolar constriction was observed when adenosine was applied in the presence of SPX. This vasoconstriction did not change significantly on superfusing the vessels with higher concentrations of adenosine.

In contrast to SPX and CPX, adenosine dilated significantly diaphragmatic arterioles in the presence of the A_2AR antagonist DMPX. However, it should be noted that dilatations observed with the highest concentration of adenosine (10^{-4} M) appeared smaller in the presence than in the absence of DMPX; this effect being more pronounced in third than in second order arterioles. However, for both orders of arterioles, this difference was not statistically significant ($P>0.05$).

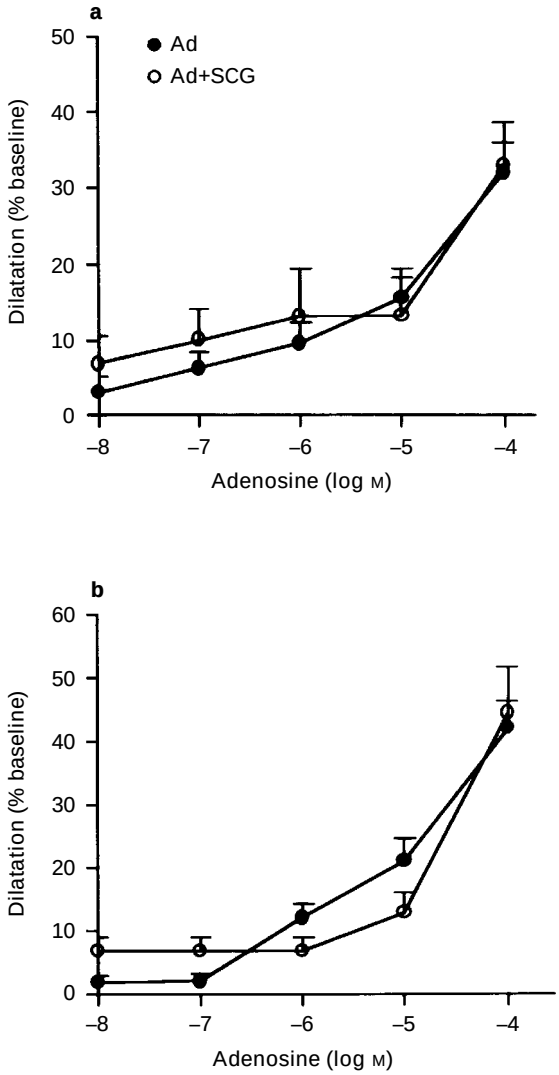

Figure 3 Concentration-response curves of (a) second and (b) third order diaphragmatic arterioles to adenosine (Ad) in absence and presence of the mast cell stabilizing agent sodium cromoglycate (SCG). $N=6$ animals and 8 and 9 second and third order arterioles, respectively. SCG did not modify significantly adenosine-induced dilatation of either order of arterioles.

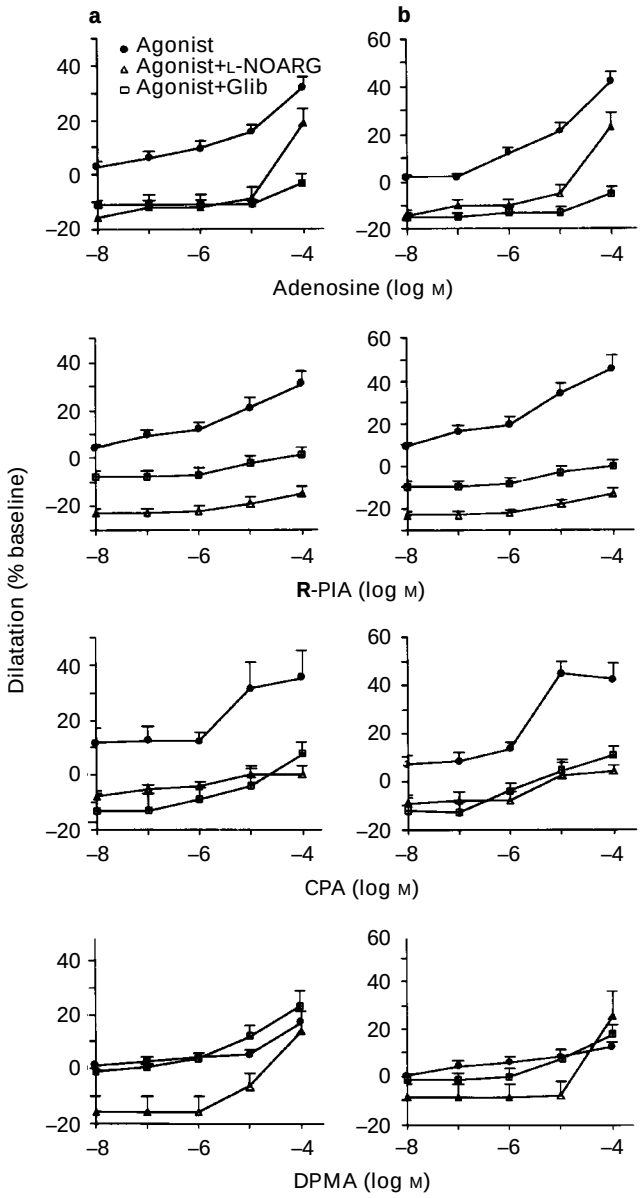

Figure 4 Effect of the NO synthesis inhibitor L-NOARG and the ATP-dependent K^+ channel blocker glibenclamide (Glib) on the concentration-response curves of (a) second and (b) third order diaphragmatic arterioles to adenosine, to the A_1AR agonists **R**-PIA and CPA, and to the A_2AR agonist DPMA. Data are expressed as mean and vertical lines show s.e.mean; $N=6$ to 12 animals and $n=10$ to 22 arterioles per group, respectively. L-NOARG inhibited significantly the vasodilatation induced by adenosine and by the agonists, in both order of arterioles. However, in contrast to **R**-PIA and CPA, the vasodilatation observed for the highest concentration of adenosine and DPMA (10^{-4} M) was not significantly reduced by L-NOARG. Glibenclamide significantly reduced diaphragmatic arteriolar dilatation induced by adenosine, **R**-PIA and CPA. In contrast, glibenclamide did not significantly modify the vasodilatation caused by DPMA.

Table 2 Diaphragmatic arteriolar diameter after superfusion of L-NOARG or glibenclamide

	Second order arterioles		*Third order arterioles*	
Group	*Diameter after L-NOARG* (% of baseline)	*Diameter after glibenclamide* (% of baseline)	*Diameter after L-NOARG* (% of baseline)	*Diameter after glibenclamide* (% of baseline)
Adenosine	84.1±4.0	88.7±1.5	85.6±2.2	84.7±1.6
R-PIA	77.4±2.1	89.5±2.4	77.3±1.5	87.0±2.6
CPA	85.1±1.8	78.6±3.8	78.5±2.9	83.8±4.4
DPMA	81.1±3.3	88.1±2.3	82.2±3.3	87.5±2.6

Data are expressed as mean±s.e.mean; $N=6$ to 12 animals and $n=10$ to 22 arterioles per group, respectively. No difference was observed between the different groups.

Role of mast cell degranulation in diaphragmatic arteriolar dilatation caused by adenosine

Figure 3 shows the effects of sodium cromoglycate on diaphragmatic arteriolar dilatation induced by adenosine. Sodium cromoglycate (10 μM) did not modify significantly the concentration-response curves of second and third order diaphragmatic arterioles to adenosine.

Role of NO in diaphragmatic arteriolar dilatation induced by adenosine and by adenosine analogues

Figure 4 shows the effects of L-NOARG (300 μM) on diaphragmatic arteriolar dilatation induced by adenosine and by the adenosine analogues **R**-PIA, CPA and DPMA. L-NOARG superfusion caused a significant reduction of second and third order arteriolar diameters. This reduction was not significantly different in the four experimental groups (adenosine, **R**-PIA, CPA and DPMA, respectively; Table 2).

L-NOARG inhibited significantly the dilatation induced by adenosine and by all the agonists, in both order of arterioles, as revealed by comparisons of concentration-response curves in the presence and in the absence of L-NOARG. However, note that in contrast **R**-PIA and CPA, the dilatation observed for the highest concentration of adenosine and DPMA (10^{-4} M) was not significantly reduced by L-NOARG.

Role of ATP-dependent K^+-channels in diaphragmatic arteriolar dilatation induced by adenosine and by adenosine analogues

Figure 4 shows the effects of glibenclamide on diaphragmatic second and third order arteriolar dilatation induced by adenosine and by adenosine analogues. As in the previous set of experiments, the effect of CGS 21680 was not evaluated. Glibenclamide superfusion caused a significant reduction of second and third order arteriolar diameters. This reduction in arteriolar diameter was not significantly different in the four experimental groups (adenosine, **R**-PIA, CPA and DPMA respectively; Table 2).

Glibenclamide inhibited significantly and similarly arteriolar dilatation induced by adenosine, **R**-PIA and CPA in both order of vessels. By contrast, glibenclamide did not significantly modify the dilatations caused by DPMA.

Discussion

The main findings of this study were: (1) the A_1AR agonists **R**-PIA and CPA dilated similarly diaphragmatic arterioles, mimicking the effect of adenosine. (2) The non selective A_2AR subtype agonist DPMA also dilated diaphragmatic arterioles but caused a significantly smaller dilatation than adenosine. By contrast the selective A_{2a}AR subtype agonist CGS 21680 did not modify diaphragmatic arteriolar diameter. (3) The A_1AR antagonist CPX abolished the effect of adenosine. By contrast, the A_2AR antagonist DMPX did not significantly alter the dilatation of diaphragmatic arterioles caused by adenosine. (4) The dilatation induced by adenosine was unchanged by the mast cell stabilizing agent sodium cromoglycate. (5) The dilatations induced by adenosine and by the A_1AR agonists were significantly attenuated by the NO synthesis inhibitor L-NOARG and by the ATP-dependent K^+ channel blocker glibenclamide. By contrast, the dilatation induced by the non-specific A_2AR subtype agonist DPMA was unchanged by glibenclamide and was only inhibited by L-NOARG when DPMA was superfused at concentrations below 10^{-4} M. These findings suggest that the A_1AR was the predominant receptor involved in the adenosine-induced dilatation of diaphragmatic arterioles. Stimulation of A_1AR induced NO synthesis and activation of ATP-dependent K^+ channels.

To our knowledge, this is the first study concerning the role of adenosine receptors in diaphragmatic microcirculation. It should be noted that the predominant role of the A_1AR in mediating diaphragmatic arteriolar dilatation induced by adenosine contrasts with the results of several *in vitro* and *in vivo* studies performed in rat brain parenchyma (Ngai & Winn, 1993), rabbit peripheral skeletal muscle (Gustafsson *et al*., 1990) and hamster skin (Stojanov & Proctor, 1989), which showed that the arteriolar vasodilator effect of adenosine was mediated exclusively through the A_2AR. It has also been suggested that A_1AR could be responsible for vasoconstriction from data obtained in porcine basilar artery (McBean, *et al*., 1988), rat kidney vessels (Rossi *et al*., 1988) and cutaneous arterioles of the hamster (Proctor & Stojanov, 1991). In two recent studies performed in the pig coronary circulation it was shown that the A_1AR can mediate vasodilatation (Merkel *et al*., 1992; Makujina *et al*., 1994). However, in these two studies the A_1ARs were not the predominant receptors involved in vasodilatation but they co-participated with the A_2AR. In the present study, the slight dilatation induced by the A_2AR agonist DPMA suggests that A_2ARs were also present in diaphragmatic arterioles. However, the lack of inhibition of adenosine induced dilatation by the A_2AR antagonist DMPX indicates that activation of these receptors plays a minor role in the effects of adenosine. Two lines of evidence suggest that stimulation of the A_2AR could co-participate with the A_1AR in the dilatation induced by high concentrations of adenosine: first, DMPX attenuated dilatation induced by the highest concentration of adenosine; second: the slight dilatation induced by the A_2AR agonist DPMA increased abruptly at the highest concentration used. Because the selective A_{2a}AR subtype agonist CGS 21680 did not modify diaphragmatic arteriolar diameter, it is likely that dilatation induced by the non-selective A_2AR subtype agonist DPMA is due to activation of A_{2b}ARs. This predominant effect of the A_{2b}AR subtype as compared to the A_{2a}AR, is in agreement with data obtained in different vascular beds, such as the pulmonary (Haynes *et al*., 1995), coronary (Abebe *et al*., 1995) and renal (Martin & Potts, 1994) circulations.

Methodological reasons could explain the difference between the present results and those obtained in other vascular beds. Adenosine tonically inhibits the release of excitatory neurotransmitters, via the A_1AR (Ribeiro, 1995). Therefore, the predominant role of the A_1AR in the present experiments could be related to a greater reduction in sympathetic tone in

the present experimental model than in other preparations. However, no data are available comparing the sympathetic tone in the present model and in the microvascular preparations cited before. Alternatively, the difference between the present results and those obtained in other vascular beds could be related to the selectivity of the adenosine analogues and antagonists used to characterize the mechanism of adenosine-induced dilatation. The selectivity of the four agonists used is well documented (see Methods for references); by contrast the selectivity of the antagonists is questionable, because it depends on the concentrations used. CPX was used at 50 nM, a concentration that has been demonstrated to be highly selective for A_1AR (more than 500 fold than for A_2AR) without effect on A_2AR (Daly *et al*., 1985; Bruns *et al*., 1987; Peet *et al*., 1990). With regard to DMPX, its affinity had been shown to be 57 times greater for the A_2AR than for the A_1AR (Seale *et al*., 1988). In the present study, DMPX was used at 10 μM, a concentration similar to that used to demonstrate involvement of A_2AR in hypoxia-induced vasodilatation of piglet pial arterioles (Park *et al*., 1995) and extensively shown to antagonize A_2AR mediated-responses in other experimental preparations (Chi *et al*., 1994; Cunha *et al*., 1995; Tabrizchi & Lupichuk, 1995). In addition, the selectivity of CPX and DMPX in our experimental conditions was determined in separate experiments. CPX was shown to be selective for A_1AR because it significantly attenuated the diaphragmatic arteriolar dilatation caused by **R**-PIA whereas it did not modify the dilatation caused by DPMA. DMPX was also shown to be selective for A_2AR because it significantly attenuated the diaphragmatic arteriolar dilatation caused by DPMA whereas it did not modify the dilatation caused by **R**-PIA. Thus, the selectivity of the adenosine antagonists used in the present study appears sufficient to warrant our conclusion that the A_1AR plays a predominant role in the adenosine-induced dilatation of diaphragmatic arterioles. Furthermore the similar abilities of the two different A_1AR agonists, **R**-PIA and CPA, to mimic the effect of adenosine, further supports this hypothesis.

It is thus likely that the difference between our results and those obtained in other vascular beds reflects heterogeneity in adenosine receptor properties between different vascular beds. This heterogeneity could result from interaction of adenosine with the parenchymal tissue cells. In fact, adenosine receptors are expressed in many cell types, such as periarteriolar mast cells, which could influence the response of the diaphragmatic arterioles to adenosine via the release of vasoconstrictor substances. In fact, Doyle and coworkers (1994) and Sheperd and associates (1996) have demonstrated *in vitro* and *in vivo* respectively, that adenosine-stimulated degranulation of periarteriolar mast cells was responsible for constriction of hamster cheek pouch arterioles, via an A_1-A_2AR-independent mechanism. However, in the case of the diaphragmatic arterioles, the role of the mast cells in modulating the effects of adenosine are less likely for the following reasons. First, no arteriole, in the present study, constricted after application of adenosine. Second, the vasoconstriction observed in the presence of the A_1-A_2AR antagonist SPX was slight (-10 to -13% of the initial diameter in the present study versus -44% in the study of Sheperd and coworkers (1996)) and did not increase significantly with increasing concentrations of adenosine, thus ruling out an A_1-A_2-AR-independent vasoconstrictive mechanism. Third, the vasodilatation induced by adenosine was not potentiated by concomitant application of the mast cell stabilizing agent sodium cromoglycate. This difference in the role of mast cells could explain heterogeneity of adenosine effects between, at least, the hamster cheek pouch and the diaphragmatic arterioles. However, because stimulation of mast cells by adenosine is not mediated by the A_1AR receptor, it is unlikely that this phenomenon could explain the predominant role of the A_1AR in diaphragmatic arterioles.

Adenosine receptors are also expressed in endothelial cells which could influence the response of diaphragmatic arterioles via the release of NO (Boczkowski *et al*., 1994). It has been recently shown that adenosine enhances NO production in endothelial cells (Li *et al*., 1995), this effect being mediated by the A_2AR (Abebe *et al*., 1995). NO produced by the endothelial cells is involved in A_2AR-mediated dilatation of the porcine (Abebe *et al*., 1995) and guinea-pig coronary circulation (Vials & Burnstock, 1993), of the guinea-pig pulmonary artery (Szentmiklosi *et al*., 1995), and of the rat renal artery (Martin & Potts, 1994). By contrast, A_1AR-mediated dilatation of porcine coronary arteries is independent of NO (Abebe *et al*., 1995). In the present study NO was also involved in adenosine-induced dilatation of diaphragmatic arterioles. In fact, both A_1AR and A_2AR mediated-dilatation involved NO. However, the dilatation induced by the highest concentration of the A_2AR agonist DPMA and by adenosine was not inhibited by L-NOARG, suggesting that this dilatation was independent of NO. The similar inhibition by L-NOARG of arteriolar dilatation induced by the two A_1AR used, **R**-PIA and CPA, showed that NO was involved in A_1AR-mediated dilatation. To our knowledge this is the first time that NO has been shown to be involved in A_1AR-mediated arteriolar dilatation. The mechanism(s) of the coupling between A_1AR stimulation and NO synthesis in diaphragmatic arterioles deserves further investigation.

Finally, the predominant role of A_1AR in mediating adenosine-induced diaphragmatic arteriolar dilatation could be also related to activation of ATP-dependent K^+ channels. Adenosine has been shown to activate ATP-dependent K^+ channels in smooth muscle from porcine coronary arteries (Dart & Standen, 1993), and to cause glibenclamide-sensitive dilatation of pig coronary arteries (Merkel *et al*., 1992) and of rat skeletal muscle vessels (Marshall *et al*., 1993). In pig coronary arteries the A_1AR is involved in the activation of these channels (Merkel *et al*., 1992). However, it should be noted that other studies have failed to find an involvement of ATP-dependent K^+ channels in adenosine-induced vasodilatation (Makujina *et al*., 1994). We determined the effects of glibenclamide, an inhibitor of ATP-dependent K^+ channels (Standen *et al*., 1989), on diaphragmatic arteriolar dilatation induced by adenosine and its analogues. Glibenclamide significantly reduced the vasorelaxation caused by the A_1AR agonists **R**-PIA and CPA, and by adenosine, but not by the A_2AR agonist DPMA. These results indicate that stimulation of the A_1AR in diaphragmatic arterioles can lead to activation of ATP-dependent K^+ channels. This could, in turn, be mediated by an increase in NO synthesis induced by A_1AR stimulation. Indeed, it has been demonstrated that NO and NO donors can activate ATP-dependent K^+ channels in smooth muscle cells (Kubo *et al*., 1994).

In conclusion, the present study showed that adenosine-induced vasodilatation of diaphragmatic arterioles was predominantly mediated by the A_1 adenosine receptors. Stimulation of the A_1 adenosine receptors induced NO synthesis and activation of ATP-dependent K^+ channels. It is also likely that the A_{2b} adenosine receptor subtype co-participates with A_1 receptors in the vasodilatation caused by high concentrations of adenosine. However, the A_{2b}AR mediated dilatation appears to be independent of NO synthesis and/or activation of ATP-dependent K^+ channels.

These findings emphasize the heterogeneity of the vascular responses to adenosine and the singularity of the diaphragmatic microcirculation. Furthermore, they present important physiological and pharmacological implications concerning the modulation of diaphragmatic blood flow, which is a major determinant of diaphragmatic muscle contractile performance.

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