

Evidence for a Role of Cyclic AMP and Endothelium in Rat Aortic Relaxation Induced by R-PIA

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Abstract: It is known that vasodilator effects of adenosine are mediated by A_2 receptors in various animal species. Nevertheless, in several blood vessels there is evidence for vasodilator responses to some adenosine analogues, which are resistant to the antagonists of the different adenosine receptor subtypes, suggesting that they are not mediated by any of the known adenosine receptors. There are contradictory reports about the effects produced by adenosine and its analogues on aortic vasodilation. The results obtained in the present study dealing with rat aortic rings and the relaxant effects induced by the selective adenosine A_1 receptor agonist N^6 -R-phenylisopropyladenosine (R-PIA) provide evidence that cyclic AMP and the presence of endothelium are possibly involved in rat aortic relaxation induced by this adenosine analogue.

Keywords: Aortic relaxation, adenosine, receptors, vasodilation, cyclic AMP, endothelium.

INTRODUCTION

It is widely known that adenosine has an important role in vasodilation in most of blood vessels [1, 2]. It has been established that relaxant responses elicited by adenosine and its analogues are mediated by A_{2A} or A_{2B} receptors [3, 4]. The vasodilator effects of adenosine in coronary vessels are mediated by A_{2A} receptors [5, 6], while adenosine-induced aortic relaxation in mice is produced through the activation of A_{2B} receptor subtype [5]. However, a number of experiments using aortic rings isolated from different animal species have shown that relaxant effects evoked by adenosine analogues are resistant to adenosine receptor antagonists [7-11], suggesting that such effects are not mediated by any of the adenosine receptors at present identified [12, 13].

Our research group, trying to find a possible attenuating effect of rat aortic relaxation mediated by A_1 adenosine receptors (the attempt was unsuccessful), confirmed the latter assumption with data indicating that rat aortic vasodilation produced by the A_1 receptor agonist N^6 -R-phenylisopropyladenosine (R-PIA), or by the A_2 receptor agonist 5'-N-ethylcarboxiamidoadenosine (NECA), can not be inhibited using A_1 , A_{2A} or A_{2B} receptors selective antagonists. There are contradictory reports about the effects of adenosine and its analogues over aortic relaxation. Mustafa and his colleagues found that the vasodilator action of adenosine is mediated by A_{2B} adenosine receptor subtype [4, 5], whilst Prentice's research group reported that adenosine dilatory effects do not involve adenosine A_1 , A_2 or A_3 receptors [8, 9, 11, 13]. These last authors also concluded that rat aortic relaxation induced by the agonist NECA, which binds

with the same affinity to A_1 and A_2 adenosine receptors, is produced through the activation of endothelial receptors to release nitric oxide (NO), which stimulates guanylate cyclase, as well as through the activation of smooth muscle receptors coupled to stimulation of adenylate cyclase, whereas neither of these mechanisms causes the vasodilator effects of R-PIA [14].

In the present work, experiments using several pharmacologic tools related to cyclic AMP signals transduction mechanism were conducted, and the results suggest that rat aortic relaxation evoked by R-PIA clearly depends, at least partially, on the increase of cyclic AMP levels, and this effect is also dependent on the presence of endothelium. Our results contrast with those described by Hourani *et al.* [14], who concluded that the vasodilation induced by R-PIA does not involve the stimulation of adenylate cyclase; they also differ from the data reported by Prentice *et al.* [13], who found that mouse aorta relaxation is independent of the presence of endothelium and nitric oxide production.

MATERIALS AND METHODOLOGY

Eleven to thirteen weeks old male Wistar rats weighing 200-250 g were sacrificed by deep anesthesia with sodium pentobarbital (100 mg/kg ip) and the thoracic aorta was gently removed and then transversely cut into rings 2-3 mm long. The rings were mounted between two steel wire hooks and suspended in 8 ml organ baths containing Krebs-Henseleit solution (in mM: NaCl, 118; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; CaCl₂·2H₂O, 2.5; D-glucose, 11), continually gassed with 95% O₂/CO₂. Aortic rings were allowed to stabilize for 60 min at a resting tension of 0.75-1 g. Tissue viability was tested using 0.1 μM phenylephrine, a concentration that elicits approximately 80% of maximum contraction [15]. All rings were tested for the presence of functional endothelium using 1 μM acetylcholine. Tissues in which acetylcholine induced less than a 25% decrease in contraction were rejected. In experiments where the effects

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of the absence of endothelium were investigated, the removal of endothelium was achieved by carefully rolling the aortic ring on a fine tungsten wire; the absence of endothelium was then confirmed by the loss of relaxant response to 1 μ M acetylcholine.

Concentration-response curves for aortic relaxation by R-PIA or NECA were obtained by cumulative addition of agonists to organ baths containing the aortic rings pre-contracted with phenylephrine (0.1 μ M) or KCl (30 mM). The concentration in the organ bath was increased in 1-log concentration steps, and agonists were added to yield the next higher concentration only when the response to the earlier dose reached a steady state. Only one concentration-response curve was constructed in each preparation. Relaxant responses were expressed as the percent decrease in the contraction induced by phenylephrine or KCl in response to each concentration of the agonist. In the experiments using antagonists, two rings were taken from the same animal, one was used as a control and one as an antagonist-treated preparation. The adenosine receptor antagonists used were: 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, A_1 receptor antagonist) (100 μ M); SCH-58261 (100 μ M) and 8-3-chlorostyryl-caffeine (CSC) (100 μ M), both A_{2A} receptor antagonists; and MRS-1754 (100 μ M) (A_{2B} receptor antagonist). The antagonists were added 60 min before contractions of the rings with phenylephrine.

For cyclic AMP signaling experiments, rings were pre-contracted with 30 mM KCl instead of phenylephrine to avoid any possible cross interaction of this adrenergic agonist with the different pharmacological tools used to study aortic relaxation induced by R-PIA. Before pre-contraction with 30 mM KCl, the aortic rings were incubated for 45 min with the following inhibitors: 100 μ M SQ-22536, 100 μ M Ro 20-1724 and 10 μ M KT-5720. Two punctual additions of dibutyl cyclic-AMP (50 μ M) were done simultaneously with the R-PIA concentration-response experiment (at 10^{-7} and 10^{-4} M R-PIA concentrations, respectively). For pertussis toxin (PTX) experiments, animals were injected with PTX (25 μ g/100 g ip) 3 days before the sacrifice to obtain the aortic segments. In all the experiments, animals were handled according to the Institutional Normative Welfare Standards (University of Aguascalientes). Signaling experiments were performed in parallel using two aortic rings from the same vessel; one served as a control (not treated) and one served as the treatment.

Changes in isometric tension were measured with a fixed-range force transducer (model R3804, Radnoti) connected to a differential amplifier (model 7173 Transducer Coupler + model 7070 Channel Amplifier, Narco-Biosystems). Data were recorded on a Hewlett Packard computer using a MP 100 WSW digital acquisition system (BIOPAC Systems) and were analyzed using Acqknowledge 3.7.4 software (BIOPAC Systems). Data are expressed as means \pm S.E.M. of values obtained from at least three different animals. Results for adenosine analogues (R-PIA and NECA) and the effects of antagonists or drugs treatment are presented as the percentage decreases in the contraction induced by phenylephrine (0.1 μ M) or KCl (30 mM). Statistical differences between means were assessed by Student's t-test and *P* values of less than 0.05 were considered significant.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used at stock concentrations of 10 mM. Phenylephrine, acetylcholine and dibutyl cyclic-AMP were dissolved in distilled water; R-PIA in 50% dimethylsulphoxide (DMSO); NECA, DPCPX, CSC, MRS-1754 and SQ-22536 in 100% DMSO; SCH-58261 in 80% DMSO; Ro 20-1724 and KT-5720 were initially dissolved in a minimal volume (40 μ l) of ethanol and methanol, and were then diluted in distilled water to obtain stock concentrations of 10mM and 1mM, respectively. Further serial dilutions were made in distilled water. The final concentrations of DMSO, ethanol and methanol in the 8 ml organ bath chamber had no effect by themselves on the aortic rings responses.

RESULTS

Cumulative relaxant curves for R-PIA and NECA were constructed in rings contracted with 0.1 μ M phenylephrine. As shown in Figs. (1) and (2), relaxant responses to R-PIA and NECA were observed at concentrations above 10^{-7} M. Fig. (1) shows that vasodilator effects induced by the A_1 receptor agonist R-PIA were not significantly affected by the treatment with DPCPX (A_1 antagonist), SCH-58261 (A_{2A} antagonist) or MRS-1754 (A_{2B} antagonist). Similarly, relaxations elicited by the agonist NECA (same affinity for A_1 and A_2 receptor subtypes) were unaffected by DPCPX, MRS-1754 or CSC (A_{2A} antagonist) (Fig. 2).

In aortic rings contracted with 30 mM KCl, the adenylate cyclase inhibitor SQ-22536 (100 μ M) produced a significant rightward shift in the concentration-response curve to R-PIA, diminishing the relaxation evoked by this agonist (Fig. 3). EC_{50} values for R-PIA increased from 1.99×10^{-5} M to 1.25×10^{-4} M in presence of the inhibitor. Maximum relaxation obtained with R-PIA (10^{-4} M) was $84.0 \pm 8.8\%$, while SQ-22536 caused a decrease to $55.7 \pm 4.9\%$ (Fig. 3). However, after removal of endothelium, SQ-22536 did not inhibit the responses to R-PIA (Fig. 4). It is important to point out that, in the absence of endothelium, R-PIA only caused a signifi-

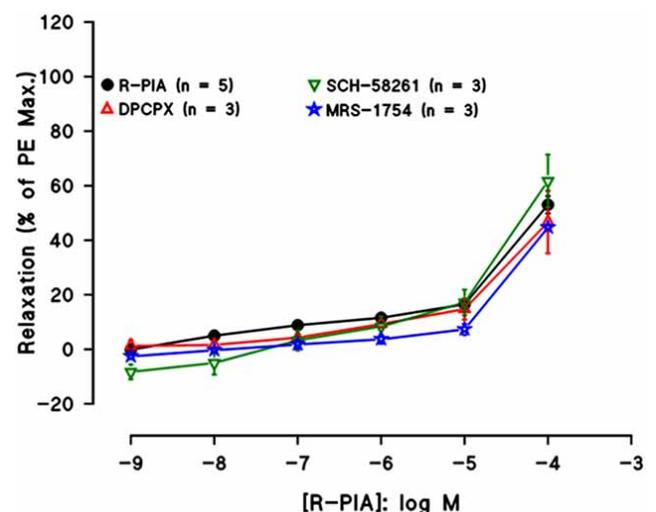


Fig. (1). Concentration-response curves to R-PIA in the absence (●) or presence of 100 μ M DPCPX (Δ), 100 μ M SCH-58261 (▽) or 100 μ M MRS-1754 (★). Each point represents the mean \pm SE (% relaxation of phenylephrine-induced contraction). For abbreviations, see text.

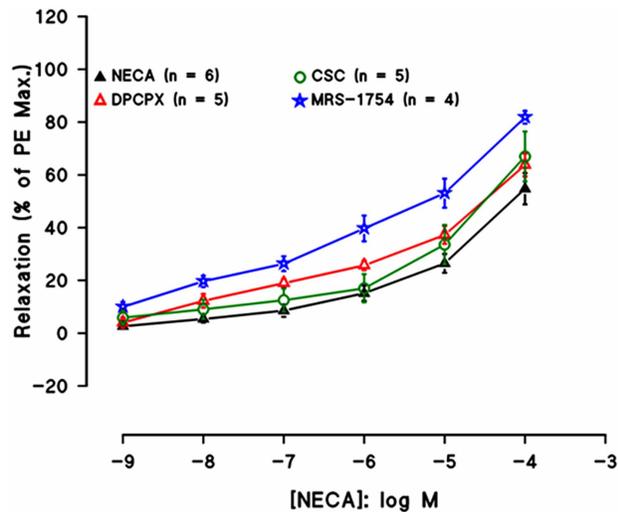


Fig. (2). Concentration-response curves to NECA in the absence (▲) or presence of 100 μM DPCPX (△), 100 μM CSC (○) or 100 μM MRS-1754 (★). Each point represents the mean ± SE (% relaxation of phenylephrine-induced contraction). For abbreviations, see text.

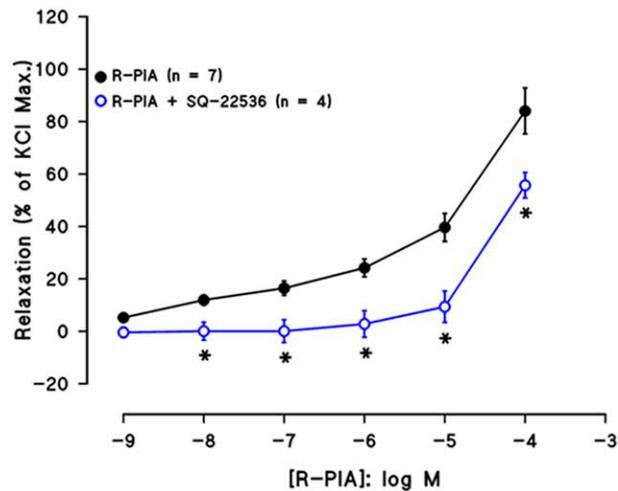


Fig. (3). Concentration-response curves to R-PIA in the absence (●) or presence of 100 μM SQ-22536 (○), in endothelium-intact rat aortic rings. Each point represents the mean ± SE (% relaxation of KCl-induced contraction). * $P < 0.05$ compared with R-PIA. For abbreviations, see text.

cant relaxant effect with the highest concentration used (10^{-4} M) (Fig. 4).

In contrast, the treatment of the aortic rings with the type IV phosphodiesterase inhibitor Ro 20-1724 (100 μM) enhanced the vasodilation elicited by R-PIA, producing a very significant leftward shift in the concentration-response curve ($EC_{50} = 9.59 \times 10^{-6}$ M in the presence of the inhibitor and $EC_{50} = 4.23 \times 10^{-5}$ M in its absence) (Fig. 5). Maximum relaxation induced by 10^{-4} M R-PIA increased from $57.5 \pm 7.0\%$ to $89.9 \pm 4.7\%$ in the presence of Ro 20-1724 (Fig. 5). Likewise, additions of the analogous of cyclic AMP: dibutyryl cyclic-AMP (50 μM) to the organ chamber also resulted in a significant leftward shift in the concentration-response curve to R-PIA, increasing its relaxing effects on

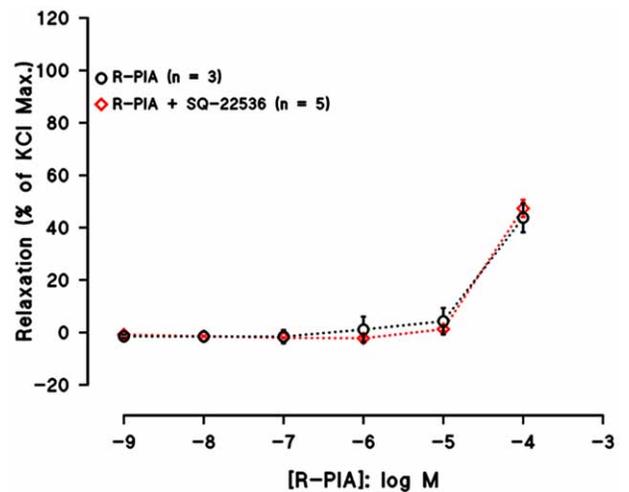


Fig. (4). Concentration-response curves to R-PIA in the absence (○) or presence of 100 μM SQ-22536 (◇), in endothelium-denuded rat aortic rings. Each point represents the mean ± SE (% relaxation of KCl-induced contraction). For abbreviations, see text.

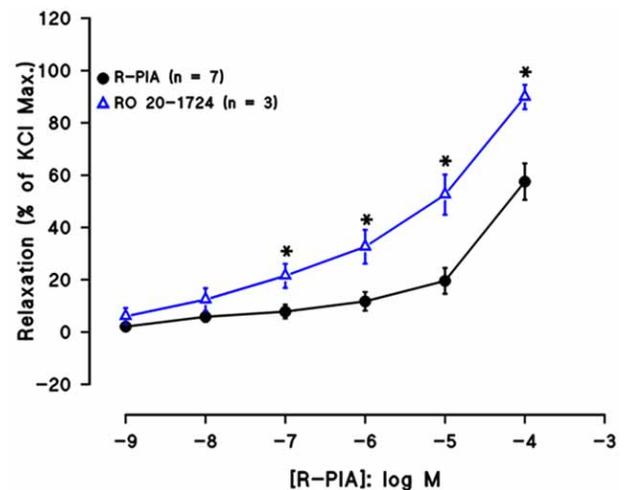


Fig. (5). Concentration-response curves to R-PIA in the absence (●) or presence of 100 μM Ro 20-1724 (△). Each point represents the mean ± SE (% relaxation of KCl-induced contraction). * $P < 0.05$ compared with R-PIA. For abbreviations, see text.

both endothelium-intact and endothelium-denuded rat aortic rings (Figs. 6 and 7). Dibutyryl cyclic-AMP also reduced EC_{50} values for R-PIA and produced significant rises of the maximum relaxations evoked by R-PIA in both preparations (Figs. 6 and 7).

Supporting the latter results, cumulative concentration-response curves constructed for R-PIA and dibutyryl cyclic-AMP show that they elicited a very similar concentration-dependent relaxation (Fig. 8), whereas the combined action of both reagents had an evident synergistic relaxing effect: EC_{50} values diminished to 2.66×10^{-5} M and the maximum relaxation increased to $109.4 \pm 3.8\%$, in comparison with relaxations induced separately by R-PIA and dibutyryl cyclic-AMP (Fig. 8).

Fig. (9) shows the enhancing effects of pertussis toxin (PTX) on vasodilation produced by R-PIA. Aortic rings isolated from animals previously injected with PTX, showed a

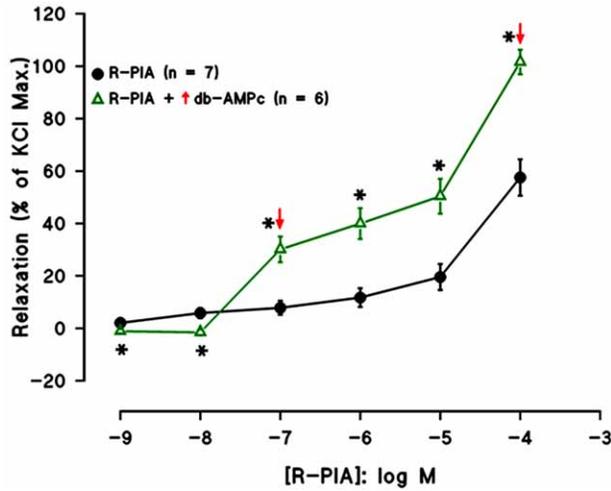


Fig. (6). Concentration-response curves to R-PIA alone (●) or with two punctual additions (▼) of 50 μM dibutyryl cyclic-AMP (Δ), in endothelium-intact rat aortic rings. Each point represents the mean ± SE (% relaxation of KCl-induced contraction). *P<0.05 compared with R-PIA. For abbreviations, see text.

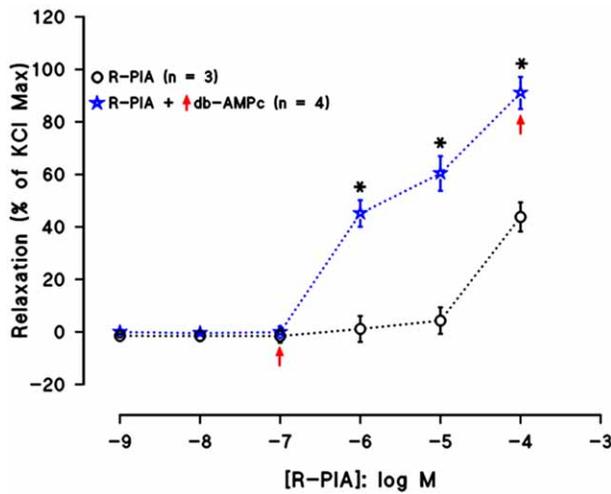


Fig. (7). Concentration-response curves to R-PIA alone (●) or with two punctual additions (▼) of 50 μM dibutyryl cyclic-AMP (Δ), in endothelium-denuded rat aortic rings. Each point represents the mean ± SE (% relaxation of KCl-induced contraction). *P<0.05 compared with R-PIA. For abbreviations, see text.

significant decrease of EC₅₀ value (from 2.73 x 10⁻⁵M to 7.93 x 10⁻⁶M) and a significant increase of the maximum relaxation evoked by R-PIA (from 71.8 ± 8.7% to 103.0 ± 5.2%), as compared with the control rings (Fig. 9). Interestingly, rings pre-incubation with the cAMP-dependent protein kinase (PKA) selective inhibitor KT-5720 (10 μM) had no significant effects on the relaxation elicited by R-PIA (Fig. 10).

DISCUSSION

Preliminary experiments originally aimed to find a possible attenuating effect of rat aortic relaxation mediated by A₁ adenosine receptors just confirmed the concepts stated in several reports, that indicate that aortic vasodilation evoked by adenosine analogues in different animal species are resis-

tant to adenosine receptor antagonists [7-11]. Our data showed that rat aortic relaxations induced by the selective adenosine A₁ receptor agonist: R-PIA, or by the A₂ receptor agonist: NECA, were resistant to blocking by A₁, A_{2A} or A_{2B} receptors selective antagonists (Figs. 1 and 2). These results are in complete agreement with those reported by Prentice *et al.* [12, 13], and, as a whole, they suggest that vasodilator effects produced by adenosine analogues in aortic rings isolated of several species, are not mediated by any of the adenosine receptors at present identified. It is reasonable to assume that such effects might be mediated by an unknown receptor activated by R-PIA and other adenosine analogues.

Therefore, experiments were conducted to determine whether the cyclic AMP signals transduction mechanism is involved in the aortic relaxation elicited by R-PIA, starting

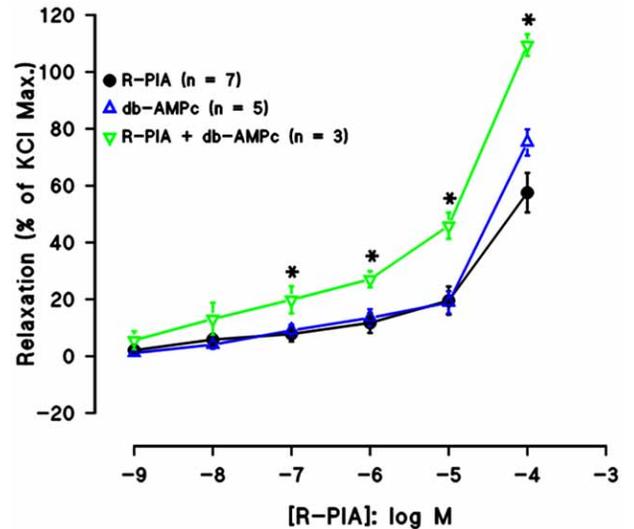


Fig. (8). Concentration-response curves to: R-PIA (●), dibutyryl cyclic-AMP (Δ), and R-PIA + dibutyryl cyclic-AMP (▽) in endothelium-intact aorta. Each point represents the mean ± SE (% relaxation of KCl-induced contraction). *P<0.05 compared with R-PIA. For abbreviations, see text.

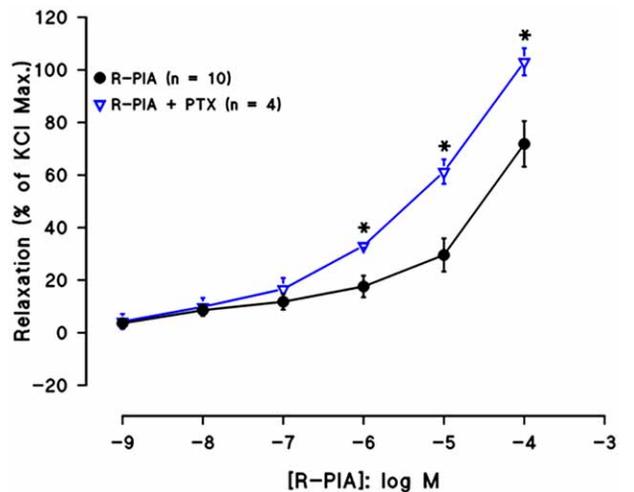


Fig. (9). Concentration-response curves to R-PIA alone (●), and to R-PIA in aortic rings isolated from rats treated with pertussis toxin (▽). Each point represents the mean ± SE (% relaxation of KCl-induced contraction). *P<0.05 compared with R-PIA. For abbreviations, see text.

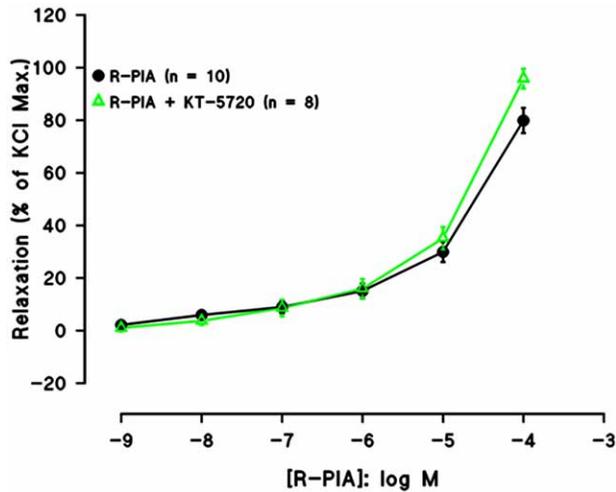


Fig. (10). Concentration-response curves to R-PIA in the absence (●) or presence of 10 μ M KT-5720 (Δ). Each point represents the mean \pm SE (% relaxation of KCl-induced contraction). For abbreviations, see text.

from the assumption that adenosine and its analogues could cause vasodilation through stimulation of adenylate cyclase and the consequent increase of cyclic AMP levels.

In endothelium-intact aortic rings, the adenylate cyclase inhibitor SQ-22536 produced a significant inhibition of the aortic relaxant effects evoked by R-PIA (Fig. 3). These results are not consistent with those reported by Hourani *et al.* [14], since they indicated a lack of effect of SQ-22536, suggesting that the stimulation of adenylate cyclase is not essential for the relaxation induced by R-PIA [14]. As shown in Fig. (3), our data clearly suggest that adenylate cyclase has an evident role in rat aortic relaxation elicited by R-PIA. The experiments using SQ-22536 in endothelium-denuded aortic rings showed that endothelium presence is necessary to allow the vasodilation produced by R-PIA, since its relaxing action at concentrations from 10^{-8} M to 10^{-5} M virtually disappeared both in control and in inhibitor-treated rings (Fig. 4). As compared with endothelium-intact rings (Fig. 3), R-PIA only induced a diminished relaxation at the higher concentration (10^{-4} M) in endothelium-denuded rings (Fig. 4).

The type IV phosphodiesterase is the enzyme responsible for degrading the cyclic AMP. The use of its inhibitor Ro 20-1724 increased the vasodilator effects evoked by R-PIA (Fig. 5), suggesting the involvement of cyclic AMP in these responses. Again, our results are slightly at variance with those reported by Hourani *et al.* [14], who only found a minor enhancement of the relaxation elicited by R-PIA in their experiments employing Ro 20-1724, although they also conclude that cyclic AMP is involved in the vasodilation produced by the agonist NECA [14]. In this respect, it could be assumed that R-PIA and NECA probably bind and activate the same hypothetical unknown receptor, since R-PIA is generally considered a selective A_1 adenosine receptor agonist, whilst the agonist NECA binds with similar affinity to A_1 and A_2 adenosine receptors, and relaxant effects induced by both agonists can not be inhibited using A_1 , A_{2A} or A_{2B} receptors selective antagonists (Figs. 1 and 2).

The results obtained with the analogous of cyclic AMP: dibutyryl cyclic-AMP, also supported the involvement of

cyclic AMP in the relaxation evoked by R-PIA, since the use of the analogous enhanced its vasodilator effects on both endothelium-intact and endothelium-denuded rat aorta (Figs. 6 and 7). Moreover, the concentration-response experiments with R-PIA and dibutyryl cyclic-AMP showed that they elicited a very similar vasodilation, while the simultaneous use of both agents had larger relaxation effects, as compared with the responses induced separately by R-PIA and dibutyryl cyclic-AMP (Fig. 8).

Finally, we examined the effects of pertussis toxin (PTX) in order to obtain additional data proving the role of cyclic AMP in the aortic relaxation elicited by R-PIA. PTX catalyzes ADP-ribosylation of G_i proteins, preventing GDP displacement by GTP and thus blocking inhibition of adenylate cyclase by G_i , causing high concentrations of cyclic AMP. As shown in Fig. 9, the pre-treatment *in vivo* with PTX produced a significant increment of relaxation, starting from a concentration of 10^{-6} M R-PIA, which substantiates the involvement of cyclic AMP levels in the vasodilator effects induced by R-PIA. This result is consistent with previous findings reported by many authors, which have widely shown that pertussis toxin relieves adenylate cyclase from the G_i inhibitory action, thus magnifying the action of agents that act through receptors coupled to G_s to stimulate the adenylate cyclase [16-18].

Altogether, the data obtained in our experiments with SQ-22536, Ro 20-1724, dibutyryl cyclic-AMP and pertussis toxin, clearly indicate the involvement of cyclic AMP in the aortic relaxation evoked by R-PIA, apparently through a mechanism which is endothelium-dependent, at least at the lower concentrations used in the assays. With the upper concentration of R-PIA, the vasodilation appears not to be endothelium-dependent, since the results using denuded aortic rings showed that 10^{-4} M R-PIA produced significant relaxant effects (Figs. 4 and 7).

However, the pre-treatment of the aortic rings with the PKA selective inhibitor KT-5720 did not affect the relaxation elicited by R-PIA (Fig. 10). Although the rest of our results strongly suggest that aortic relaxation induced by R-PIA depends on cyclic AMP levels, the data obtained using KT-5720 imply that PKA might not be involved in the vasodilation. In this respect, Hourani *et al.* indicated that vasodilator effects induced by NECA are endothelium-dependent and that this agonist acts at least partially through the endothelial release of nitric oxide (NO) [14]. These authors propose a model in which NECA acts through A_{2A} receptors to cause both direct stimulation of adenylate cyclase in smooth muscle and stimulation of NO release from endothelial cells which causes an increase in cyclic GMP in the smooth muscle [14]. They also quoted the information obtained by other authors, in order to explain their results about rat aortic relaxation produced by NECA, and they remark that endothelium-dependent effects of NECA and the release of NO were possibly associated with the stimulation of an endothelial adenylate cyclase and the increase in cyclic AMP levels [14]. The latter assumption could also be a likely explanation for the aortic vasodilation evoked by R-PIA observed in our experiments.

According to the model proposed by Hourani *et al.* [14], our results could be explained assuming that aortic relaxation

induced by R-PIA is due to the activation of a receptor located in endothelial cells, which in its turn stimulates adenylyl cyclase (see our data with SQ-22536), increasing cyclic AMP levels to produce the aortic relaxation observed, but not through the activation of a cyclic AMP-dependent protein kinase (see our data with KT-5720); as the model of Hourani *et al.* [14] suggests for NECA induced relaxation, R-PIA could act through the stimulation of endothelial receptors to release NO, which stimulates guanylate cyclase to increase cyclic GMP in the smooth muscle. Nevertheless, the results shown in Fig. (7) do not support this assumption, since they indicate that cyclic AMP alone is capable to cause aortic relaxation in absence of endothelium. Therefore, further experiments assaying several pharmacological tools related to NO signaling pathway are necessary to determine its implication in rat aortic vasodilation evoked by R-PIA.

In summary, two major conclusions can be drawn from the present study: first, the rat aortic relaxation induced by R-PIA depends, at least partially, on cyclic AMP levels; and second, the receptor activated by R-PIA seems to be mainly located in endothelial cells. The activation of this endothelial receptor by R-PIA would stimulate adenylyl cyclase with a consequent increase of cyclic AMP levels.

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