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Effects of P_1 and P_{2Y} purinoceptor antagonists on endothelium-dependent and -independent relaxations of rat mesenteric artery to GTP and guanosine

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1 Guanosine 5'-triphosphate (GTP) and guanosine can relax both endothelium-intact and -denuded arterial preparations. In the present work the P_1 and P_{2Y} purinoceptor antagonists, 8-phenyltheophylline and reactive blue 2, respectively, were used to study the mechanisms of relaxation responses induced by GTP, guanosine, adenosine 5'-triphosphate (ATP) and adenosine in noradrenaline-precontracted rat mesenteric artery rings.

2 GTP (10 μM–1 mM) dose-dependently relaxed endothelium-intact mesenteric artery rings and also induced moderate relaxation responses in endothelium-denuded preparations. Pretreatment of the rings with 8-phenyltheophylline (10 μM) or reactive blue 2 (10 μM) did not attenuate the relaxant effect of GTP.

3 Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a dose-dependent manner. The presence of 8-phenyltheophylline or reactive blue 2 had no effects on guanosine-induced relaxations.

4 ATP-induced (0.1 μM–0.1 mM) relaxation of endothelium-intact artery rings was attenuated by reactive blue 2 while 8-phenyltheophylline was ineffective. ATP also relaxed endothelium-denuded artery rings and this relaxation was inhibited by 8-phenyltheophylline, but not by reactive blue 2.

5 Adenosine-induced (10 μM–1 mM) relaxation of endothelium-intact and -denuded artery rings was attenuated by the presence of 8-phenyltheophylline, but not of reactive blue 2.

6 In conclusion, the endothelium-dependent and -independent relaxations of rat mesenteric arteries to GTP and guanosine are not mediated via P_1 and P_{2Y} purinoceptors. Therefore, these results support our previous suggestion on the presence of a novel guanine nucleotide-specific receptor, a putative P_G receptor, on both endothelial and smooth muscle cells, which may participate in the regulation of arterial tone.

Keywords: Endothelium; vascular tone; guanine nucleotides; adenine nucleotides; purinoceptors; purinoceptor antagonists

Introduction

Extracellular adenosine and adenine nucleotides modulate vascular tone and platelet function by interacting with specific receptors on the cell surface, ATP and ADP acting at P_2 purinoceptors and adenosine at P_1 purinoceptors (Burnstock, 1978; Burnstock & Kennedy, 1985; Gordon, 1986; Olsson & Pearson, 1990). ATP and ADP can be liberated into the extracellular space as a consequence of vessel wall damage and local platelet aggregation (Gordon, 1986), after which they are rapidly sequentially dephosphorylated to adenosine by ectonucleotidases of endothelium and circulating blood cells (Coade & Pearson, 1989). The endothelium-independent effect of ATP can be either vasodilatation or vasoconstriction mainly depending on the subclass of the P_2 purinoceptor in question (Kennedy *et al.*, 1985; Houston *et al.*, 1987; Pearson, 1988). The endothelium-dependent effect of ATP is vasodilatation due to its action at P_{2Y} purinoceptors which leads to release of endothelium-derived relaxing factor (EDRF-NO) and/or prostacyclin (De Mey & Vanhoutte, 1981; Gordon & Martin, 1983; Needham *et al.*, 1987; Boeynaems & Pearson, 1990). It is suggested that the vasodilator action of adenosine is mediated via the A_2 subclass of P_1 purinoceptors and stimulation of adenylate cyclase in smooth muscle (Collis & Brown, 1983; Ramagopal *et al.*, 1988). The location of adenosine receptors has not been well-defined and both endothelium-dependent (Gordon & Martin, 1983; Rubanyi & Vanhoutte, 1985) and -independent (Kennedy *et al.*, 1985; White & Angus, 1987) responses have been described.

Naturally occuring xanthines, such as theophylline and caffeine, are P_1 receptor antagonists (Olsson & Pearson, 1990). Alkylxanthine derivatives, such as 8-phenyltheophylline, are nonselective, but equally potent antagonists of A_1 and A_2 purinoceptors, and can be used as tools to study adenosine-mediated mechanisms (Bruns *et al.*, 1983).

The anthraquinone sulphonic acid derivative, reactive blue 2, which can be regarded as an ATP analogue, has been used as a P_{2Y} antagonist, which over a narrow concentration-range selectively antagonizes P_{2Y}-mediated dilator responses to ATP in various blood vessels (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Houston *et al.*, 1987; Reilly *et al.*, 1987).

Although the vasoactive effects of extracellular adenine nucleotides are well characterized, there is little information about the effects of extracellular guanine nucleotides on vascular tone. We have recently reported that exogenous GTP and guanosine (i) induce smooth muscle relaxation (ii) promote accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) without affecting the levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and (iii) augment the effects of nitrovasodilators in both endothelium-intact and -denuded rat mesenteric artery rings (Vuorinen *et al.*, 1991; 1992). The endothelium-dependent response to GTP of endothelium-intact rings can be explained by the subsequent release of EDRF-NO, whereas that induced by guanosine is

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only partially mediated via autacoid release from the endothelium. Moreover, the endothelium-independent responses to GTP and guanosine are direct, unknown effects of these compounds on smooth muscle and guanylyl cyclase (Vuorinen *et al.*, 1992). The present work was carried out to examine the effects of the P_1 and P_{2Y} purinoceptor antagonists, 8-phenyltheophylline and reactive blue 2, respectively, on endothelium-dependent and -independent relaxations to exogenous GTP and guanosine to obtain more evidence for the existence of novel purinoceptors specific for guanine nucleotides. The known purinoceptor agonists, ATP and adenosine, were used as reference compounds.

Methods

Relaxation of rat mesenteric arteries

Non-fasted male Sprague-Dawley rats weighing about 300 g were decapitated. The mesenteric artery was excised and cut into 3 mm long rings. Six rings were usually obtained from one artery. When endothelium-dependent effects were studied, the endothelium was left intact but for studies of endothelium-denuded rings the endothelium was removed by rubbing it gently with a jagged injection needle. The rings were placed between two stainless steel hooks and mounted in an organ bath chamber containing Krebs bicarbonate buffer solution (pH 7.4) of the following composition (in mM): NaCl 119.0, $NaHCO_3$, 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2. The preparations were maintained at 37°C and aerated with 95% O_2 and 5% CO_2 and before the experiments they were equilibrated for 1 h with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer (Grass FT03) and registered on a Grass Polygraph (Model 7 E Polygraph; Grass Instrument Co., Quincy, MA, USA).

Successful removal or the integrity of the endothelium was confirmed by adding acetylcholine (1 μM, final concentration) to 0.5 μM noradrenaline-precontracted vascular rings. If any relaxation of the denuded preparation was observed, the endothelium was further rubbed. For studies with endothelium-intact vascular rings, the relaxation with acetylcholine had to be nearly 100%. The absence or presence of the endothelium when applying the present methods has been confirmed in our previous study by electron microscopy, showing undamaged underlying smooth muscle cells in denuded preparations and a well preserved endothelial cell monolayer in intact rings (Arvola *et al.*, 1992).

After rinsing four times with Krebs buffer the rings were stabilized for an additional 30 min. Thereafter cumulative vascular relaxation responses to GTP (10 μM–1 mM), guanosine (10 μM–1 mM), ATP (0.1 μM–1 mM) or adenosine (10 μM–1 mM) were elicited. The rings were precontracted with 0.5 μM noradrenaline, and after the contraction had fully developed increasing concentrations of relaxing agents were added cumulatively. The relaxation time for each dose was 5 min but for the highest dose it was 10 min. The rings were then washed four times with Krebs buffer and allowed to return to baseline tension. After 30 min 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 was added, and 15 min later the rings were again contracted with noradrenaline and the cumulative relaxations were repeated in the presence of antagonist. Only one of the above purines was used to study the relaxation responses in each vascular ring and the concentrations and incubation periods were chosen on the basis of our own pilot studies.

Drugs

Acetylcholine chloride, GTP, ATP, guanosine, adenosine, 8-phenyltheophylline and reactive blue 2 were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). (-)-Noradrenaline-L-hydrogen tartrate was from Fluka Chemie AG (Buchs, Switzerland). The test agents were prepared in Krebs buffer on the day of use.

Statistical analysis

The results are expressed as mean ± s.e.mean. Statistical analysis was carried out by one-way analysis of variance (ANOVA) for repeated measurements. The data were analysed with BMDP Statistical Software (Los Angeles, CA., U.S.A.). P values of less than 0.05 were considered to be statistically significant.

Results

The effects of 8-phenyltheophylline and reactive blue 2 on endothelium-dependent and -independent relaxations to GTP and guanosine

GTP (10 μM–1 mM) dose-dependently relaxed noradrenaline-precontracted endothelium-intact and -denuded artery rings. The relaxation at 1 mM GTP was 99 ± 1% in intact and 35 ± 1% in denuded rings (Figure 1). Pretreatment of the rings with 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 for 15 min before inducing contraction did not attenuate the relaxant effect of GTP (Figure 1).

Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a concentration-dependent manner. The relaxation to 1 mM guanosine was 73 ± 3% in

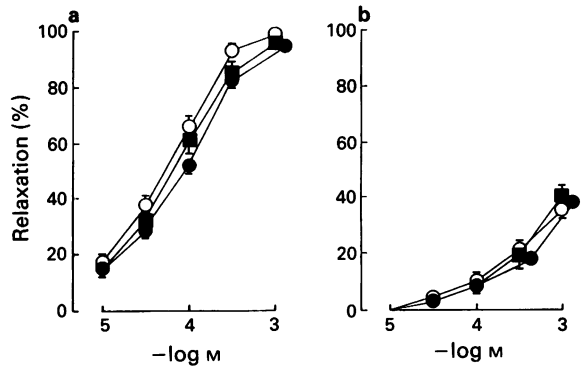

Figure 1 Concentration-response curves for GTP in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean (n = 6–12 rings of 4–6 rats).

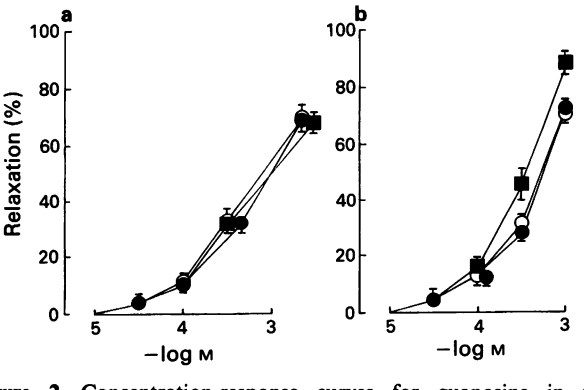

Figure 2 Concentration-response curves for guanosine in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean (n = 6–12 rings of 4–6 rats).

intact and 70 ± 3% in denuded rings (Figure 2). The presence of 10 μM 8-phenyltheophylline or 10 μM reactive blue 2 had no significant effects on guanosine-induced relaxations. 8-Phenyltheophylline even tended to augment the relaxation in denuded rings (Figure 2b).

The effects of 8-phenyltheophylline and reactive blue 2 on endothelium-dependent and -independent relaxations to ATP and adenosine

ATP (0.1 μM–0.1 mM) efficiently relaxed endothelium-intact artery rings in a dose-dependent manner, the relaxation at 0.1 mM of ATP being 97 ± 1% (Figure 3a). The presence of 10 μM reactive blue 2 significantly ($P<0.01$) attenuated ATP-induced relaxation, but 10 μM 8-phenyltheophylline had no effect (Figure 3a). ATP also relaxed endothelium-denuded rings at higher concentrations (10 μM–1 mM), the relaxation at 1 mM ATP being 89 ± 3% (Figure 3b). Pretreatment with 10 μM reactive blue 2 did not affect ATP-induced relaxation of denuded rings, but 10 μM 8-phenyltheophylline had an inhibitory effect ($P<0.01$) (Figure 3b).

Adenosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a concentration-dependent manner, the relaxations being slightly more pronounced in intact rings. The relaxation induced by 1 mM adenosine was 88 ± 3% in intact and 81 ± 3% in denuded rings (Figure 4). The presence of 10 μM 8-phenyltheophylline attenuated ($P<0.01$) adenosine-induced relaxations in both types of vascular preparations, while reactive blue 2 had no significant effects (Figure 4).

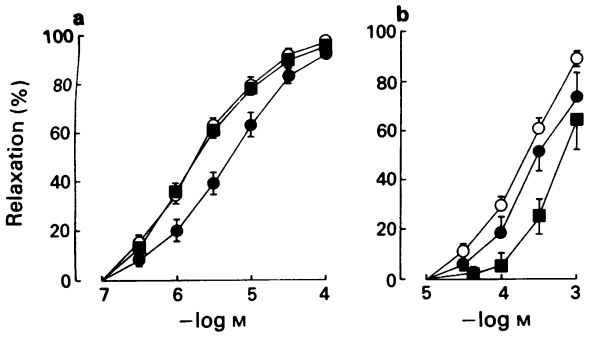

Figure 3 Concentration-response curves for ATP in the absence (○) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e. mean ($n = 6–12$ rings of 4–6 rats). $P<0.01$ (ANOVA) in intact rings when comparing the relaxation to ATP in the absence and presence of reactive blue 2; $P<0.01$ (ANOVA) in denuded rings between the responses to ATP in the absence and presence of 8-phenyltheophylline.

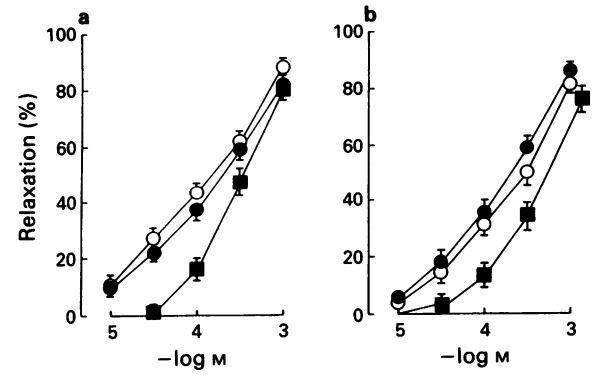

Figure 4 Concentration-response curves for adenosine in the absence (●) and presence of 10 μM 8-phenyltheophylline (■) and 10 μM reactive blue 2 (●) in endothelium-intact (a) and endothelium-denuded (b) rat mesenteric artery rings. Values are the mean ± s.e.mean ($n = 6–12$ rings of 4–6 rats). $P<0.01$ (ANOVA) in both intact and denuded rings when comparing the relaxations to adenosine in the absence and presence of 8-phenyltheophylline.

Discussion

In the present study 8-phenyltheophylline and reactive blue 2 were used as tools to examine whether GTP- and guanosine-induced relaxations of rat mesenteric arteries are mediated via P_1 and P_{2Y} purinoceptor activation. The well characterized purinoceptor agonists, ATP and adenosine, were used as control relaxants to confirm the efficacy of the antagonists in this experimental model. However, the results obtained with these purinoceptor antagonists as investigative tools must be interpreted with some caution. Xanthines are not particularly potent and selective for A_1 and A_2 subtypes of P_1 receptors, although the C-8 substitution with phenyl increases the potency but not the selectivity of the compound (Collis *et al.*, 1985). Reactive blue 2 is a specific and selective P_{2Y} antagonist but only over a narrow concentration-range (Olsson & Pearson, 1990), and when used at higher concentrations it has nonspecific effects, for example, an attenuating influence on A_2 receptor-mediated vasodilatation (Hopwood & Burnstock, 1987).

8-Phenyltheophylline was able to attenuate the adenosine-induced relaxations both in endothelium-intact and -denuded arterial rings. Thus, the present results agree with previous findings that adenosine is a direct vasodilator inducing vascular relaxation via activation of P_1 purinoceptors on smooth muscle, and that the presence of an intact endothelium is not required for its relaxant effect on arteries (Furchgott, 1984). 8-Phenyltheophylline also inhibited the relaxation of denuded preparations to ATP, suggesting that this endothelium-independent response of rat mesenteric arteries is due to the production of adenosine from ATP by ectonucleotidases during the incubation period. A similar mechanism has previously been reported to be operative in pulmonary vessels and thoracic aorta of the rat (Liu *et al.*, 1988; Rose'Meyer & Hope, 1990).

In a variety of arteries, including the rat mesenteric bed (Ralevic & Burnstock, 1988), dog coronary artery (Houston *et al.*, 1987), pig aorta (Martin *et al.*, 1985) and rat femoral artery (Kennedy *et al.*, 1985) the P_{2Y} purinoceptor-mediated relaxation induced by ATP and its analogues has been shown to be endothelium-dependent and presumably to occur via the subsequent release of EDRF-NO. In the present study, reactive blue 2 attenuated the ATP-induced relaxation of endothelium-intact rat mesenteric rings thus indicating the inhibition of P_{2Y} receptor activation and the subsequent release of EDRF-NO. Previously we have shown that the ATP-induced relaxation of rat mesenteric rings correlates with an increase in smooth muscle cyclic GMP concentration (Vuorinen *et al.*, 1992).

The applied P_1 and P_{2Y} antagonists, 8-phenyltheophylline and reactive blue 2, respectively, which inhibited the ATP- and adenosine-induced relaxations in an expected manner, did not affect the endothelium-dependent and -independent relaxations of rat mesenteric arteries to GTP and guanosine. We have earlier shown that exogenous GTP and guanosine relax precontracted endothelium-intact and -denuded rat mesenteric artery rings by increasing cyclic GMP accumulation without affecting cyclic AMP concentration in smooth muscle (Vuorinen *et al.*, 1992). Moreover, the GTP-induced relaxation of endothelium-intact rings was attenuated by N^G-nitro L-arginine methyl ester (L-NAME), an effect which could be reversed by L-arginine, but the guanosine-induced relaxation of endothelium-intact rings was only slightly inhibited by L-NAME (Vuorinen *et al.*, 1992). Thus, the response to GTP of endothelium-intact rings could mainly be explained by the release of EDRF-NO, but that of guanosine was only partly due to EDRF-NO. The endothelium-

independent response of GTP and guanosine is a direct, unknown effect on smooth muscle and guanylyl cyclase.

We have recently reported that exogenous GTP can elevate cyclic GMP concentration in human ADP-stimulated platelet rich plasma and in thrombin-stimulated washed platelets (Laustiola *et al.*, 1991; Vuorinen & Laustiola, 1992). This increase in cyclic GMP was accompanied by decreased platelet aggregation. The stimulation of platelets leads to secretion of nucleotides from granules, where ATP and ADP are stored at concentrations up to one molar (Gordon, 1986). The concentration of guanine nucleotides, mainly GTP and GDP, in granules is about 30% of that of adenine nucleotides (Holmsen, 1985). Therefore, during platelet stimulation and secretion, local GTP concentrations can increase enough to produce an inhibition of platelet function. Taken together, the results on platelets and smooth muscle suggest that GTP may have a local antithrombotic effect via elevation of cyclic GMP. Thus, GTP could regulate platelet-vessel wall interaction even when endothelial function is impaired.

In conclusion, the endothelium-dependent effect of GTP and the putative EDRF-NO-release are not mediated via P_1 or P_{2Y} activation, and the endothelium-independent relaxation to GTP and the response to guanosine are not due to activation of adenosine-specific P_1 purinoceptors. Therefore, the present results support our earlier suggestions on the presence of a cell membrane site of action, of a novel guanine nucleotide specific receptor (a putative P_G receptor) which can mediate the activation of soluble guanylyl cyclase and participate in the regulation of arterial tone.

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