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Effects of pyrimidines on the guinea-pig coronary vasculature

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1 The effects of the pyrimidines, uridine 5'-triphosphate (UTP), thymidine 5'-triphosphate (TTP) and cytidine 5'-triphosphate (CTP), were examined in the guinea-pig coronary bed, by use of a Langendorff technique. Comparisons were made with the actions of the purines adenosine 5'-triphosphate (ATP), inosine 5'-triphosphate (ITP) and guanosine 5'-triphosphate (GTP). The effect of, the nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME) and, the prostaglandin synthesis inhibitor, indomethacin on the vasodilator response to these purines and pyrimidines was examined. The effects of these inhibitors were assessed on their ability to inhibit both the amplitude and the area of the vasodilator response.

2 The relative order of potency of the purines and pyrimidines studied was ATP > UTP > ITP >> GTP, TTP, CTP.

3 The maximum amplitude and area of the vasodilator response to the pyrimidines, UTP (5×10^{-10}–5×10^{-7} mol), TTP (5×10^{-8}–5×10^{-7} mol) and CTP (5×10^{-7} mol), and purines, ITP (5×10^{-9}–5×10^{-7} mol) and GTP (5×10^{-8}–5×10^{-7} mol), were significantly reduced by L-NAME (3×10^{-5} and 10^{-4} M).

4 The inhibition of the response to ATP (5×10^{-8} mol), UTP (5×10^{-8} mol), ITP (5×10^{-8} mol), TTP (5×10^{-7} mol), CTP (5×10^{-7} mol) and GTP (5×10^{-7} mol) by L-NAME (3×10^{-5} M) was significantly reversed by L-arginine (1.5×10^{-3} M).

5 L-NAME (3×10^{-5} and 10^{-4} M) only inhibited the amplitude of the vasodilator response to a low dose of ATP (5×10^{-10} mol), although the area of vasodilator response to ATP (5×10^{-11}–5×10^{-7} mol) was significantly reduced by L-NAME (3×10^{-5} and 10^{-4} M).

6 The maximum amplitude of the vasodilator response to ATP (5×10^{-10}–5×10^{-7} mol) was significantly reduced by indomethacin (10^{-6} M), although the area of the vasodilator response to ATP was only significantly reduced at one intermediate dose (5×10^{-9} mol). Indomethacin (10^{-6} M) did not affect the maximum amplitude or area of the vasodilator responses to UTP (5×10^{-11}–5×10^{-7} mol), ITP (5×10^{-10}–5×10^{-7} mol), CTP (5×10^{-7} mol), TTP (5×10^{-8}–5×10^{-7} mol) and GTP (5×10^{-8}–5×10^{-7} mol).

7 It is concluded that in the guinea-pig coronary vasculature, the vasodilatation evoked by the pyrimidines, UTP, TTP and CTP, was mediated in large part via nitric oxide, as were the vasodilatations evoked by the purines ITP and GTP. The vasodilatations evoked by ATP, however, appear to involve prostanoids in addition to the release of nitric oxide.

Keywords: Nitric oxide synthase; L-N^G-nitroarginine methyl ester; prostanoids; indomethacin; coronary vasculature; relaxation of smooth muscle; purines; pyrimidines

Introduction

Adenosine 5'-triphosphate (ATP) produces powerful systemic effects; it influences many biological processes being released from nerve endings, platelets and endothelial cells in physiological and pathophysiological processes (Burnstock & Kennedy, 1986). In the cardiovascular system its ability to cause vasoconstriction or vasodilatation is mediated through activation of subtypes of P_2-purinoceptor (Burnstock & Kennedy, 1985; Hoyle, 1992). In the rat coronary vasculature, ATP causes vasoconstriction via P_{2X}-purinoceptors and vasodilatation via P_{2Y}-purinoceptors (Hopwood & Burnstock, 1987). The naturally occurring nucleotides, cytidine 5'-triphosphate (CTP), thymidine 5'-triphosphate (TTP) and uridine 5'-triphosphate (UTP) which are pyrimidines, and guanosine 5'-triphosphate (GTP), which is a purine, have also been shown to have effects on the vasculature. Of these, probably the most studied has been UTP which may be released from blood platelets (Goetz *et al.*, 1971; Urquilla, 1978). In many tissues, including the piglet aorta (Martin *et al.*, 1985), 2-methylthioATP (2-meSATP) is a potent agonist producing a similar maximum relaxant response to ATP. The relative order of agonist potencies of these two compounds is consistent with that conventionally described for P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985): 2-meSATP >> ATP = ADP >> α,β-methyleneATP (α,β-meATP), UTP. However there is also a variety of tissues in which ATP causes phospholipase C activation but to which the above agonist potency order does not apply; because of the common second messenger system in these tissues they have been loosely linked with the P_{2Y}-subtype (Kennedy, 1990; Boeynaems & Pearson, 1990). In these tissues 2-meSATP has little or no activity, for example, although ATP induces prostaglandin I_2 (PGI_2) production in bovine aortic smooth muscle cells, 2-meSATP does not (Demolle *et al.*, 1988). This indicates that there is a subpopulation of phospholipase C-linked P_2-purinoceptors that are insensitive to 2-meSATP. As such, these sites cannot correctly be classified as P_{2Y}-purinoceptors. This pattern is strengthened by the observation that UTP has similar agonist potency to ATP in many of the tissues that are unresponsive to 2-meSATP. Davidson and colleagues (1990) introduced the term 'nucleotide' receptor for the ATP/UTP-sensitive site on sheep pituitary cells. This convention was adopted and it was proposed that a nucleotide receptor may be characterized by the following agonist potency order: UTP = ATP > ADP > α,β-meATP, 2-meSATP (O'Connor *et al.*, 1991). Tissues like rat aorta (Dainty *et al.*, 1990) may contain a heterogeneous population of receptor types (possibly both P_{2Y} and 'nucleotide' recep-

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tors), activation of which results in the same functional response, in this case endothelium-dependent relaxation. To support this it has recently been demonstrated that two separate co-existing receptor populations (P_{2Y}-purinoceptors and nucleotide receptors) are located on bovine aortic endothelial cells (Motte *et al.*, 1993; Wilkinson *et al.*, 1993).

Endothelial cells play a key role in the control of vascular tone by virtue of their ability to synthesize and release endothelium-derived relaxing factors. Adenosine 5'-triphosphate has been shown to elicit vasodilatation in the coronary bed via an action at receptors located on endothelial cells, leading to release of these factors (Hopwood & Burnstock, 1987; Hopwood *et al.*, 1989). These factors include prostacyclin (Moncada & Vane, 1979) and endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980). Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) which can be released from endothelial cells by a variety of stimuli including UTP and ATP (Pearson *et al.*, 1983; Demolle *et al.*, 1988). In the guinea-pig coronary vasculature the release of EDRF, believed to be nitric oxide (NO, Ignarro *et al.*, 1986; Furchgott *et al.*, 1987) mediates relaxation evoked by ATP (Vials & Burnstock, 1992) and in the rat mesenteric arterial bed UTP also induces relaxation via NO (Ralevic & Burnstock, 1991).

This study investigates the relaxant effects of pyrimidine nucleoside triphosphates UTP, CTP and TTP and to compare them with the relaxant effects of the purine nucleoside triphosphates ATP, inosine 5'-triphosphate (ITP) and GTP, on the guinea-pig coronary vasculature. Inhibitors of the enzymatic synthesis of NO and prostaglandins are used, namely L-N^G-nitroarginine methyl ester (L-NAME) which is a competitive inhibitor of the synthesis of NO from L-arginine (Rees *et al.*, 1990) and is effective in inhibiting vasodilator responses to various agents including ATP in the guinea-pig coronary vasculature (Vials & Burnstock, 1992), and indomethacin which is a prostaglandin synthesis inhibitor (Vane, 1971).

Methods

Guinea-pigs (250–400 g) of either sex were injected with heparin (2 500 units i.p.) 15 min before being killed by cervical dislocation. The heart was removed and placed in cold Krebs solution (4°C) to arrest the beating. Extraneous fat and large vessels were removed, the heart was cannulated via the aorta, and the coronary circulation perfused by the method of Langendorff with a modified Krebs-Henseleit solution containing (mM): NaCl 115.3, KCl 4.6, $MgSO_4$. $7H_2O$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5 and glucose 11.1. Albumin (0.5 g l^{-1}) was also added to the solution to increase the oncotic pressure and reduce oedema. A water-filled silicone rubber balloon, connected to a pressure transducer (Viggo-Spectramed Bilthoven, model P23XL), was placed in the left ventricle for the measurement of left ventricular pressure. The left ventricular diastolic pressure did not exceed 10 mmHg. The average starting left ventricular systolic pressure was 41.92 ± 1.21 mmHg ($n = 57$). Perfusion pressure was monitored with a pressure transducer connected via a side arm to the cannula. A pair of platinum electrodes were placed in the right ventricle and the heart was paced at 4 Hz with electrical pulses of 5 ms duration at supramaximal voltage (usually around 20 V). The flow rate was gradually increased to obtain a starting perfusion pressure of 50–60 mmHg, using a Masterflex constant flow roller pump (Cole-Palmer Instruments Co., Chicago). The flow rate was determined by collecting the effluent, over a period of time, and the average rate was 13.26 ± 0.35 ml min^{-1}($n = 57$). The heart was left to equilibrate for at least 20 min before commencing the experiment.

When the perfusion had reached a steady state, the purines and pyrimidines were given as a bolus of 50 µl, injected over 3 s into the superfusing solution close to the heart. The duration of each individual experiment was no longer than 3 h. Due to this time restriction the effects of all the agonists could not be tested on the same heart. For this reason the agonists were chosen randomly and not more than four agonists were used on a particular heart. The order of exposure of the agonists to the heart was also random to minimize effects due to time-dependent changes and preparation variability. At least 5 min was left between the administration of each dose of agonist. When the effect of antagonists was examined, control dose-response relationships for the purines and pyrimidines were first obtained and L-NAME or indomethacin added to the perfusing solution and allowed to equilibrate for 20 min. The dose-responses were then repeated in the presence of the antagonist. After inhibition with L-NAME, L-arginine was also added to the perfusing solution to determine whether the inhibition could be reversed. The preparations were allowed to equilibrate for a further 20 min before a submaximal dose of agonist was repeated. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis programme on an Apple II Computer. Student's t tests were used to assess statistical significance between responses obtained before and after antagonist, $P < 0.05$ being taken as significant. The rank order of potency of the purines and pyrimidines was determined empirically as maximum responses to these agents were not obtained and therefore pD_2 values could not be calculated. At the end of the experiment the heart was removed from the cannula, blotted and weighed. The mean wet weights were 1.46 ± 0.04 g ($n = 57$).

ATP, ITP, UTP, GTP, TTP, CTP, L-NAME, L-arginine and indomethacin were all obtained from Sigma Chemical Co. Ltd. Indomethacin was made up as a stock solution (10^{-2} M) in sodium carbonate solution (10^{-2} M). The remaining compounds were made up as stock solutions (10^{-1} M) in distilled water. A 50 µl bolus injection of distilled water caused no change in perfusion pressure other than a small injection artefact.

To test for the presence of ATP as a contaminant of GTP, TTP, CTP and ITP, solutions (10^{-2} M) of these agents were assayed for ATP with the luciferin-luciferase technique described by Stanley & Williams (1969). A negligible amount of ATP was found in any of these solutions.

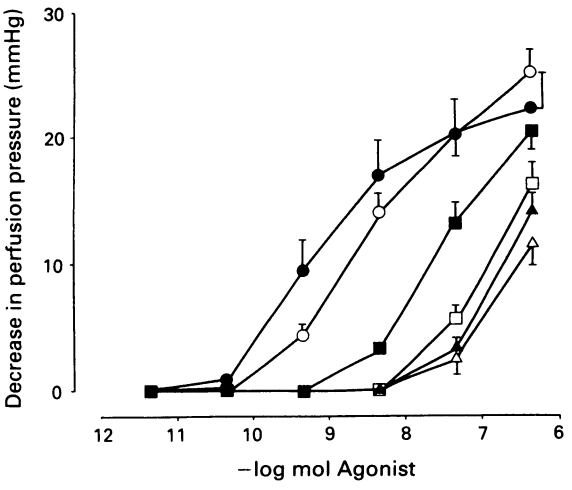

Figure 1 The amplitude of the vasodilatation evoked by adenosine 5'-triphosphate (●), uridine 5'-triphosphate (○), inosine 5'-triphosphate (■), thymidine 5'-triphosphate (□), guanosine 5'-trisphosphate (▲) and cytidine 5'-triphosphate (△) in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 8$.

Results

Dose-response to pyrimidines and purines

Bolus injections of ATP, UTP, ITP, GTP, CTP, and TTP produced dose-dependent vasodilatation in the guinea-pig coronary vasculature. Dose-response curves for the six agonists are illustrated in Figure 1. Due to the fact that maximum responses to these agents were not obtained, an arbitrary decrease in perfusion pressure of 10 mmHg was used to determine the relative order of potency. The potency order of these agonists was ATP > UTP >> ITP >> TTP, GTP, CTP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. There was a small but insignificant fall in the left ventricular systolic pressure on bolus administration of agonists at the high doses.

Effect of L-NAME and L-arginine

The effect of L-NAME on the maximum amplitude of the vasodilator response (Figure 2a–c), on perfusion pressure trace response (Figure 5) and on the area (Figure 3a–c) of the vasodilator response to ATP, UTP and ITP is demonstrated. The maximum amplitude and area of the vasodilator responses due to UTP (5×10^{-10}–5×10^{-7} mol) and ITP (5×10^{-9}–5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; Figures 2a,b, 3a,b and Figure 5a). L-NAME (3×10^{-5} and 10^{-4} M) only inhibited the amplitude of the vasodilator response to a low dose of ATP (5×10^{-10} mol; Figure 2c). In contrast, the area of the vasodilator response to ATP (5×10^{-11}–5×10^{-7} mol; Figures 3c and 5b) was significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M), reflecting an attenuation of the duration of the response. The maximum amplitude and area of

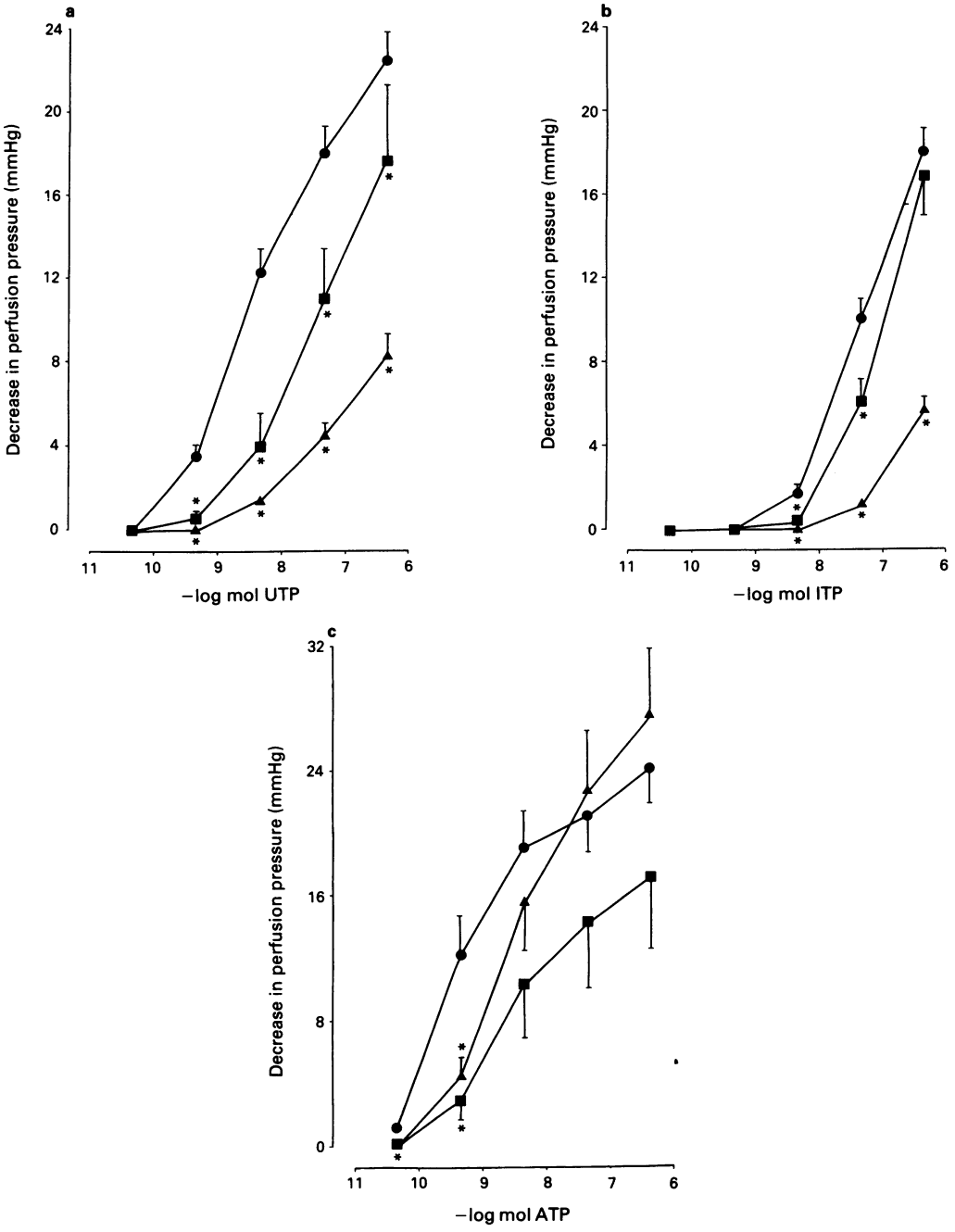

Figure 2 The amplitude of the vasodilatation obtained in response to (a) uridine 5'-triphosphate, (b) inosine 5'-triphosphate and (c) adenosine 5'-triphosphate, in the absence (●, mean of all controls) or presence of L-N^G-nitroarginine methyl ester (3×10^{-5} M (■) and 10^{-4} M (▲)), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences from control are $^*P < 0.05$.

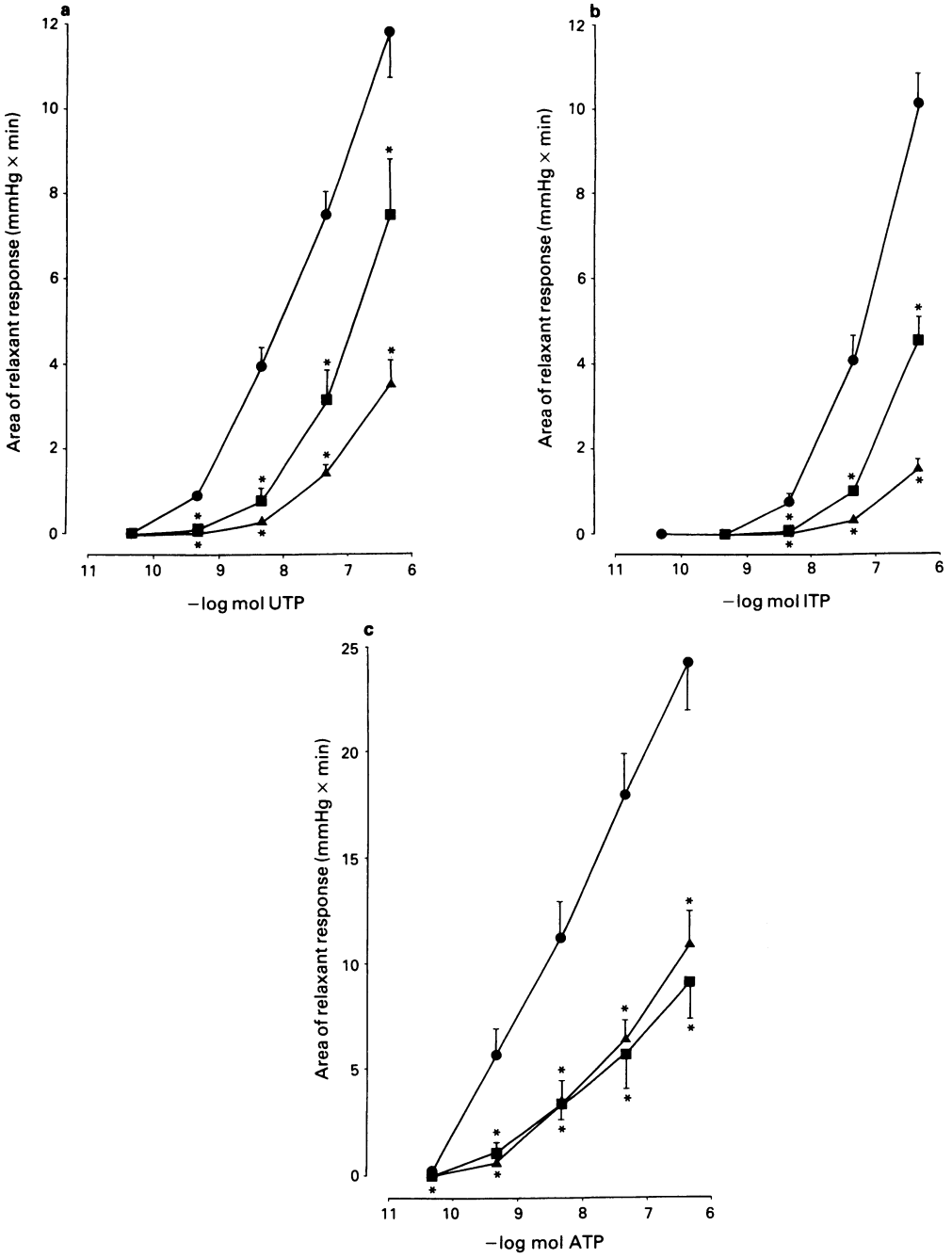

Figure 3 The area of the vasodilatation obtained in response to (a) uridine 5′-triphosphate, (b) inosine 5′-triphosphate and (c) adenosine 5′-triphosphate, in the absence (●, mean of all controls) or presence of L-N^G-nitroarginine methyl ester (3×10^{-5} M (■) and 10^{-4} M (▲)), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences from control are $^*P < 0.05$.

the vasodilator responses to GTP (5×10^{-8}–5×10^{-7} mol), CTP (5×10^{-7} mol) and TTP (5×10^{-8}–5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; data not shown). The inhibition of the response to ATP (5×10^{-8} mol), UTP (5×10^{-8} mol), ITP (5×10^{-8} mol), TTP (5×10^{-7} mol), CTP (5×10^{-7} mol) and GTP (5×10^{-7} mol) by L-NAME (3×10^{-5} M) was significantly reversed by L-arginine (1.5×10^{-3} M; Table 1 and Figure 5a,b). L-NAME (3×10^{-5} and 10^{-4} M) and L-arginine (1.5×10^{-3} M) did not significantly affect the resting perfusion pressure or left ventricular pressure of the preparations.

Effect of indomethacin

Indomethacin (10^{-6} M) did not affect the maximum amplitude or area of the vasodilator responses to UTP (5×10^{-11}–5×10^{-7} mol; Figures 4a and 5a), ITP (5×10^{-10}–5×10^{-7} mol; Figure 4b), CTP (5×10^{-7} mol; data not shown), TTP (5×10^{-8}–5×10^{-7} mol; data not shown) and GTP (5×10^{-8}–5×10^{-7} mol; data not shown). In contrast, the maximum amplitude of the vasodilator response to ATP (5×10^{-10}–5×10^{-7} mol; Figures 4c and 5b) was significantly reduced by indomethacin (10^{-6} M). The area of the response to ATP was only significantly reduced at one intermediate dose (5×10^{-9} mol; data not shown). The resting perfusion pressure and left ventricular pressure of the preparations were unaffected by the addition of indomethacin (10^{-6} M).

Discussion

The results of this study revealed that the rank order of potency of the pyrimidines and purines in the guinea-pig

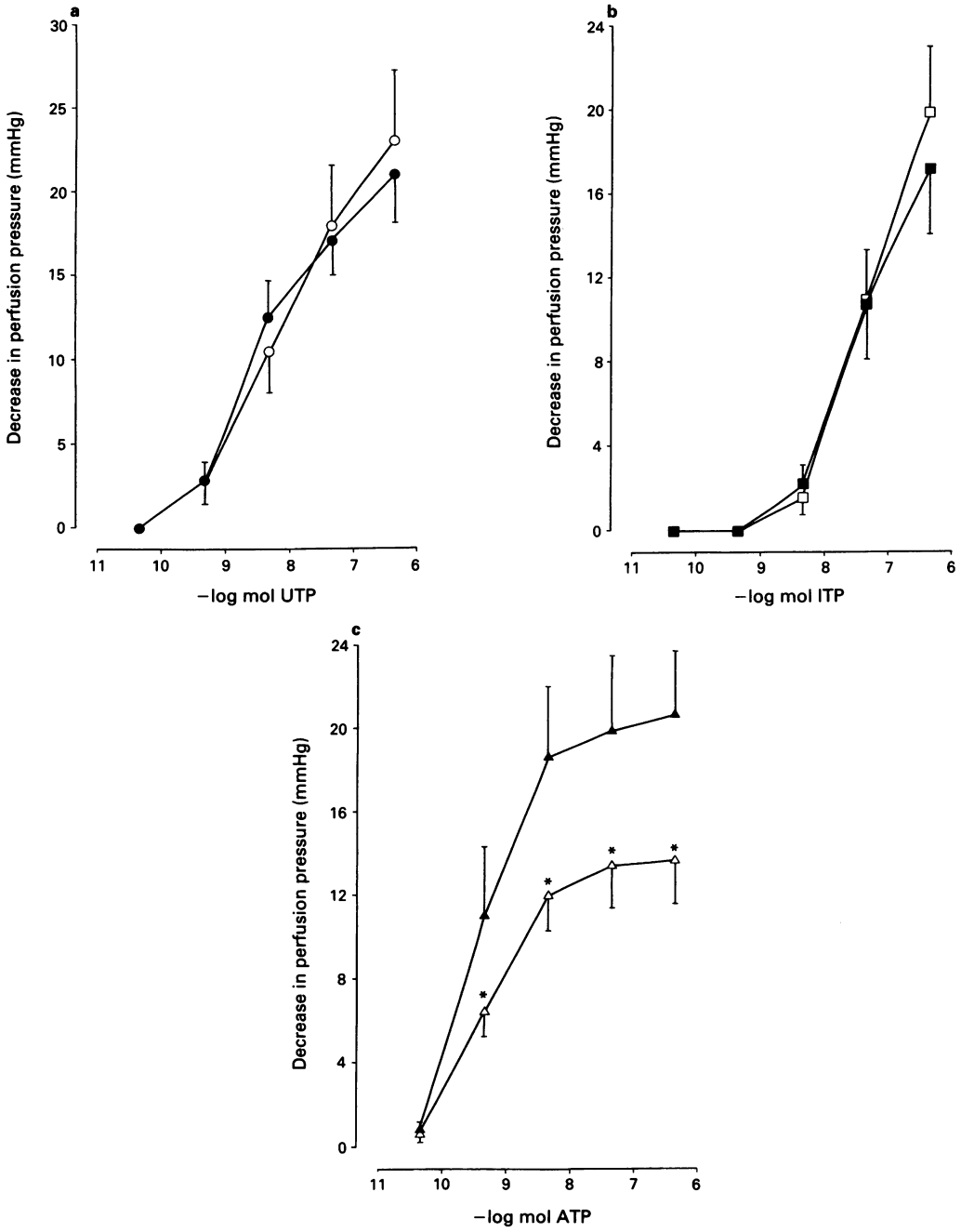

Figure 4 The amplitude of the vasodilator responses evoked by (a) uridine 5'-triphosphate, (b) inosine 5'-triphosphate and (c) adenosine 5'-triphosphate, in the absence (closed symbol) or presence (open symbol) of indomethacin (10^{-6} M), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences are $^{*}P < 0.05$.

coronary vasculature was ATP > UTP > ITP >> GTP, TTP, CTP. The pyrimidines UTP, CTP and TTP induce relaxation in a similar way to the purine compounds ITP and GTP in that the vasodilator responses to these pyrimidines and purines were dependent largely upon the synthesis of NO. In contrast, vasodilator responses evoked by ATP were only partially dependent upon the synthesis of NO. Prostanoids also play a role in the relaxation induced by ATP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. Contamination of these compounds could explain the responses obtained to these agents. Although minimal amounts of ATP were detected in solutions (10^{-2} M) of these compounds there is always the possibility that UTP is the contaminant.

It has been shown, in the rat mesenteric arterial bed, that relaxations induced by ATP, TTP, UTP and GTP are dependent upon an intact endothelium (Ralevic & Burnstock, 1991). In the pig aorta (Martin *et al.*, 1985) and human pial vessels (Hardebo *et al.*, 1987) relaxation to UTP is also endothelium-dependent. ATP can stimulate prostanoid production from perfused vascular beds and from endothelial cells in culture (Pearson & Gordon, 1985) and in the guinea-pig coronary vasculature it has been shown to induce release of NO (Kelm & Schrader, 1990). UTP has also been shown to stimulate prostacyclin production in endothelial cells (Forsberg *et al.*, 1987) and in the perfused rat liver (Haussinger *et al.*, 1988). In the rat mesenteric arterial bed, vasodilatation to UTP is in large part due to release of NO (Ralevic & Burnstock, 1991). We used this information to investigate and possibly to distinguish between the vascular mechanisms of pyrimidines and purines by using L-NAME, an inhibitor of the conversion of L-arginine to NO (Rees *et al.*, 1990), and indomethacin, a prostaglandin-synthesis inhibitor (Vane, 1971). A more direct approach to distinguish

Table 1 The area of the relaxation obtained in response to adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), inosine 5'-triphosphate (ITP), cytidine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP) and thymidine 5'-triphosphate (TTP) in the guinea-pig isolated perfused heart. The effect of L-N^G-nitroarginine methyl ester (L-NAME (3×10^{-5} M) in the perfusate) on the response to the agonists and the effect of L-arginine (L-Arg (1.5×10^{-3} M) in the perfusate along with L-NAME) on the inhibition by L-NAME is shown

		Area of relaxant response (mmHg × *min*)		
Agonist	*Dose (mol)*	*Control*	*After addition of: L-NAME*	*L-NAME + L-Arg*
ATP	5×10^{-8}	22.32 ± 3.40	10.53 ± 2.45*	20.85 ± 5.00**
UTP	5×10^{-8}	8.30 ± 1.13	3.14 ± 0.75*	5.63 ± 1.18**
ITP	5×10^{-8}	6.24 ± 1.27	1.00 ± 0.18*	4.10 ± 1.17**
CTP	5×10^{-7}	2.87 ± 0.78	0.03 ± 0.03*	1.74 ± 0.66**
GTP	5×10^{-7}	3.61 ± 0.40	0.83 ± 0.16*	3.58 ± 0.66**
TTP	5×10^{-7}	6.90 ± 0.98	1.49 ± 0.34*	3.37 ± 0.36**

The areas are expressed as the mean ± s.e.mean ($n \geq 6$). Significant differences from control are $*P < 0.05$. Significant differences from responses obtained in presence of L-NAME are $**P < 0.05$.

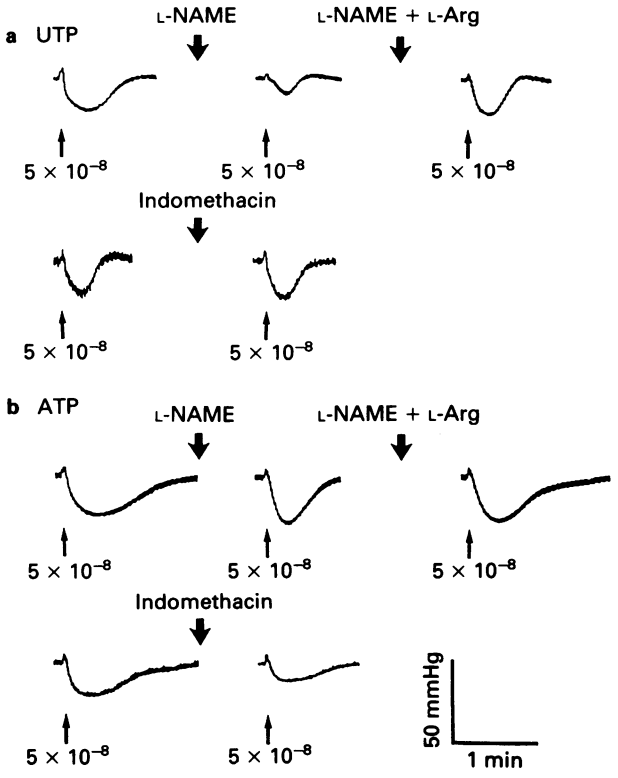

Figure 5 Typical perfusion pressure traces, obtained from guinea-pig isolated perfused hearts, showing the effects of (a) UTP and (b) ATP. The vasodilator response to these agents in the absence or presence (after ↓) of L-N^G-nitroarginine methyl ester (L-NAME, 3×10^{-5} M) and indomethacin (10^{-6} M) is demonstrated. Reversal of the inhibition of the vasodilator response to UTP and ATP, in the presence of L-NAME, by L-arginine (L-Arg, 1.5×10^{-3} M) is also demonstrated. The dose stated is the number of mol of vasodilator agonist that is injected into the perfusion system close to the heart.

between P_{2Y}-purinoceptor-mediated and 'nucleotide' receptor-mediated relaxations could not be adopted because of the absence of specific antagonists to the 'nucleotide' receptor and because the P_{2Y}-purinoceptor antagonist, reactive blue 2 (Burnstock & Warland, 1987), caused rapid deterioration of the tissue.

The maximum amplitude and area of the vasodilator responses to the pyrimidines, UTP, CTP and TTP, and the purines, ITP and GTP, were significantly reduced by L-NAME. These results suggest that the relaxant response to UTP, CTP, TTP, ITP and GTP take place largely through the formation and release of NO. In the rat mesenteric arterial bed, the vasodilator response to UTP is also largely dependent on the release of NO (Ralevic & Burnstock, 1991). The fact that L-arginine reversed the inhibition of the response to the pyrimidines and purines by L-NAME substantiates these claims in that it shows the L-NAME was selectively inhibiting the enzyme nitric oxide synthase. Nitric oxide synthase converts L-arginine into L-citrulline with the additional production of NO (Palmer *et al.*, 1988; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). It therefore appears that the pyrimidines and purines studied induce relaxation in a similar manner with the exception of ATP. In the guinea-pig coronary vasculature it has been shown that ATP induces release of NO (Kelm & Schrader, 1990). However, as previously demonstrated (Vials & Burnstock, 1992), while L-NAME reduced the duration of the vasodilatation induced by ATP, it did not alter the peak response, suggesting that at least this part of the response is not due to the generation and release of NO.

Indomethacin, the prostaglandin synthesis inhibitor (Vane, 1971), significantly reduced the maximum amplitude of the vasodilator response to ATP. This suggests that prostanoids are also involved in part of the response to ATP. ATP has been shown to stimulate prostacyclin production from various beds and endothelial cells in culture (Needleman *et al.*, 1974; Boeynaems & Galand, 1983; Hellewell & Pearson, 1984). In the guinea-pig coronary vasculature, adenosine-induced relaxation was not mediated via prostanoids (Vials & Burnstock, 1993). Therefore the involvement of prostanoids in the relaxant response to ATP was not due to its breakdown to adenosine by highly active ectonucleotidases (Fleetwood *et al.*, 1989). The vasodilator responses to the pyrimidines UTP, TTP and CTP or the purines ITP and GTP were unaffected by the presence of indomethacin. Therefore prostanoids do not play a role in the vasodilatation produced in response to exposure to these pyrimidines and purines. In contrast UTP has been shown to induce prostacyclin production from bovine pulmonary artery endothelial cells (Lustig *et al.*, 1992).

In conclusion, we have demonstrated that the pyrimidines, UTP, TTP and CTP, and purines, ITP and GTP, induce relaxation in the guinea-pig coronary bed via formation and release of NO. ATP induces relaxation in the guinea-pig coronary vasculature via a combination of mechanisms involving both NO and prostanoids. Whether 'nucleotide' receptors are also present in the guinea-pig coronary vasculature is unclear. If they were present then action at these receptors induces relaxation via NO and not prostanoids. Selective antagonists will need to be established before a clear receptor profile in the guinea-pig coronary vasculature can be determined.

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