The role of the endothelium in mediating the actions of ATP, adenosine and acetylcholine on flow through blood vessels in the rabbit knee joint

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- 1 An *in vitro* preparation of the rabbit knee joint, perfused with oxygenated Locke solution, was used to investigate the presence of purinoceptors and the role of endothelium within articular blood vessels.
- 2 The basal tone of the blood vessels was not affected by adenosine or acetylcholine. Adenosine 5'-triphosphate (ATP) injection produced vasoconstriction which was unaffected by removal of the endothelial layer, but diminished by α , β methylene ATP, a compound which desensitizes P₂-purinoceptors.
- 3 When knee joint blood vessel tone was raised by perfusion with vasopressin (10^{-8} M) or 5-hydroxytryptamine (10^{-5} M), acetylcholine, ATP and adenosine were all found to induce concentration-dependent relaxation of these vessels. ATP was found to have a dual effect of transient constriction followed by longer-lasting dilatation.
- 4 3-Methylxanthine, a P₁-purinoceptor antagonist significantly reduced the relaxation response to adenosine but had no effect on the vasodilator effect of ATP.
- 5 Removal of the endothelial layer virtually abolished the vasodilator effects of acetylcholine and ATP but not adenosine.
- 6 These results demonstrate that articular blood vessels supplying the rabbit knee contain P₁-purinoceptors located on the vascular smooth muscle which mediate vasodilatation. P₂-purinoceptors mediating a constrictor effect are also present on this smooth muscle. It is likely that the vasodilator effect of ATP is mediated via P₂-purinoceptors located on the endothelial layer.

Introduction

Despite the prevalence of inflammatory joint disease, relatively little is known about the factors which influence blood flow to joints. Although there have been numerous *in vitro* and *in vivo* studies of blood vessels in a variety of tissues which have characterized their responses to different agents, little is known about articular blood vessels.

Cobbold & Lewis (1956) found that close intra-arterial injection of adrenaline and noradrenaline both produced vasoconstriction of blood vessels in the canine knee joint. This finding has recently been confirmed in a more extensive and quantitative investigation of rabbit knee joint blood vessels using different α - and β -adrenoceptor agonists and antagonists (Ferrell & Khoshbaten, 1989). β -Adrenoceptors were found to be absent and the vasoconstrictor effects of the catecholamines were mediated by α_1 -adrenoceptors.

In recent years attention has been focussed on purine nucleotides and nucleosides which have been found to have widespread and potent vascular actions (Haddy & Scott, 1968; Burnstock, 1980; Su, 1981; Burnstock *et al.*, 1984). Some of these actions appear to be mediated via the endothelium as Furchgott (1981) found that removal of the endothelial layer of dog arteries abolished the vasodilator action of acetylcholine (ACh), adenosine 5'-triphosphate (ATP), ADP, substance P and bradykinin. Subsequently, it was shown that ATP, but not adenosine, induced vasodilatation via a purinoceptor located on the endothelium in many different blood vessels (De Mey & Vanhoutte, 1981; 1982; 1983; Vanhoutte & Rimele, 1983; Burnstock & Kennedy, 1986). However, other studies have found that the vasodilator action of adenosine can be mediated via the endothelium (Gordon & Martin, 1983; Frank & Bevan, 1983). Kennedy & Burnstock (1985) and Burnstock & Kennedy (1986) have shown that ATP can produce vasoconstriction via P₂-purinoceptors located on vascular smooth muscle cells.

The aim of these experiments was to assess the types of purinoceptors present on articular blood vessels and whether or not any effects of ATP, adenosine and acetylcholine are mediated via the endothelium.

Methods

White New Zealand rabbits of either sex weighing between 2–2.3 kg, were killed by a blow to the skull followed by exsanguination. Immediately thereafter, the posterior aspect of the knee joint was exposed, the popliteal artery cannulated and its muscular branches ligated as described by Ferrell & Khoshbaten (1989). The tissue was perfused with warmed and oxygenated Locke solution by means of a peristaltic pump (Gilson Minipuls). The knee was separated from the animal by sawing through the femur above the point of cannulation. The lower part of the limb was also removed by sawing mid-way through the tibia. The isolated knee joint preparation was then transferred to a thermostatically controlled bath ($37 \pm 1^\circ\text{C}$) containing oxygenated Locke solution. At the start of the experiment the pump was set to provide a perfusion rate of $0.5\text{--}0.8\text{ ml min}^{-1}$ which resulted in a perfusion pressure of about 40–50 mmHg as measured by a pressure transducer connected 'downstream' from the pump. After a 30 min equilibration period a steady resting pressure was achieved and subsequent changes in perfusion pressure provided an indirect means of assessing articular blood vessel calibre. Peak response was compared to control (pre-injection) value and expressed as percentage change from control (or baseline). 'Control' injections of Locke solution were administered periodically and were found to produce little change in pressure, apart from a transient rise during the injection phase.

Vascular tone was increased by the addition of vasopressin 10^{-8} M or 5-hydroxytryptamine (5-HT) 10^{-5} M or 10^{-6} M to the perfusate. These resulted in perfusion pressure increases of about 160–200% of control (100%) values.

Removal of the endothelial layer in these preparations was achieved by perfusion with $1\text{--}3\text{ mg ml}^{-1}$ solution of sodium

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Figure 1 Photomicrographs of blood vessels from the rabbit knee joint. (a) Normal appearance of a muscular artery; inset shows a higher power view of the area outlined, with endothelial cells (E) lining the internal elastic lamina (IE). Haemalum and eosin. (b) After perfusion with deoxycholate, endothelial cells no longer line the blood vessel. Inset shows the internal elastic lamina (IE) intact but an absence of endothelial cells. Toluidine blue.

deoxycholate, in Locke solution for 30 s on two separate occasions 1 min apart (Byfield *et al.*, 1986). As illustrated in Figure 1, this procedure was effective in disrupting the endothelial layer in five preparations tested.

The composition of Locke solution was (mm): NaCl 115, KCl 4.7, NaHCO₃ 24.1, CaCl₂ 2.5, MgSO₄ · 7H₂O 1.2, KH₂PO₄ 1.2 and glucose 5.6. CaCl₂ was added after oxygenating the solution with 95% O₂ and 5% CO₂ mixture. Compounds at different concentrations were administered as a bolus (0.2 ml) injection into tubing beyond the pump. In the case of antagonists, these were added to Locke solution which was then perfused continuously. The following agents were administered: [Arg⁸]-vasopressin acetate salt, adenosine, adenosine 5'-triphosphate (sodium salt), α , β methyleneadenosine 5'-triphosphate (lithium salt), 5-hydroxytryptamine HCl (5-HT), deoxycholic acid (sodium salt), acetylcholine HCl, 3-methylxanthine, and atropine sulphate, all from Sigma. All drugs were dissolved in Locke solution.

Statistical data analysis was by either paired or unpaired *t* test. An *F* test was used to test the assumption of homogeneity of variances. Where this exceeded tabled *F* values, modified *t* values were generated using the formula described by Phillips (1978). All responses have been expressed as the means $\pm$ s.e.mean. Differences between means were considered significant if the *P* values were 5% or less (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). In all graphs data are presented as mean $\pm$ s.e.mean.

Results

Basal vascular tone

Intra-arterial injection of ATP in doses ranging from 0.02 to 2 μ M produced vasoconstriction of articular blood vessels

Figure 2 (a) Trace showing the effect of injection of Locke solution, adenosine and ATP on the perfusion pressure. CNT indicates the control pressure prior to injection. The interval between each dot on the timescale indicates 1 min. (b) Dose-response relationship for adenosine (■) and ATP (□). Locke indicates response to injection of Locke solution. ****P* < 0.001. In both (a) and (b) the responses were obtained with basal vascular tone. For adenosine *n* = 7 at each dose, and for ATP *n* = 8–11.

(Figure 2a) which was dose-dependent (Figure 2b). Maximum vasoconstriction was usually achieved within a minute, but was not maintained (Figure 2a). Almost no desensitization was observed, even when the drugs were re-administered after a short interval.

Exogenous adenosine (0.02 to 2 nM) had no vasoconstrictor effect on knee joint blood vessels (Figure 2a,b) but a slight and transient dilator effect was observed at 2 nM. In a few preparations higher doses of adenosine were tried but no further vasodilatation occurred. Acetylcholine (ACh, 0.2 to 20 nM) also failed to constrict these vessels except in two preparations where ACh did elevate perfusion pressure when administered as a single dose. Perfusion with α , β methylene ATP transiently increased the perfusion pressure but this was not sustained. None of the above constrictor responses was reduced by removal of endothelium.

Raised vascular tone

Vascular tone was raised by the addition of either vasopressin 10⁻⁸ M (before ACh injection) or 5-HT 10⁻⁵ M or 10⁻⁶ M (before purine injections) to the perfusate. Adenosine in doses from 0.02 to 2 nM and ATP (0.02 to 2 μ M) were then found to produce concentration-dependent relaxation of articular blood vessels (Figure 3b). The dual effect of ATP is illustrated (Figure 3a), consisting of a transient constriction followed by a long-lasting dilatation. Only rarely was the initial constrictor response absent.

Perfusion of the preparation with 3-methylxanthine (10⁻⁵ M), a P₁-purinoceptor antagonist (Burnstock, 1978), did not affect the vasodilator action of ATP but abolished the response to injected adenosine (Figure 3b). The dilator action

Figure 3 (a) Trace showing the dual effect of ATP on joint blood vessels after elevation of vascular tone by perfusion with 5-hydroxytryptamine (10^{-6} M). CNT represents the perfusion pressure prior to raising blood vessel tone. Small dots represent 1 min intervals. (b) Vasodilator effect of adenosine (●) and ATP (■) on blood vessels with raised tone. During perfusion with the P_1 -purinoceptor antagonist 3-methylxanthine (10^{-5} M), the response to adenosine (○) was abolished ($n = 7$) but the response to ATP (□) was unaffected ($n = 6$).

of adenosine was unaffected by removal of the endothelial layer suggesting that this is mediated via P_1 -purinoceptors on the smooth muscle cells of the knee joint blood vessels. The vasoconstrictor effect of ATP was mediated via P_2 -purinoceptors as perfusion of articular blood vessels with

Figure 5 The constrictor response to injected ATP before (●) and after removal of the endothelial layer by sodium deoxycholate pretreatment (○). The dot-dash line shows the response to ATP during perfusion with 3-methylxanthine (10^{-5} M). In both cases vascular tone was elevated by perfusion with 5-hydroxytryptamine (10^{-5} M). $n = 6-7$.

α, β methylene ATP, a potent P_2 -purinoceptor desensitizing agent, abolished the vasoconstrictor effect of ATP (Figure 4a). This vasoconstriction was unaffected by perfusion with 3-methylxanthine but was slightly increased after removal of the endothelial layer (Figure 5).

The vasodilator action of ATP was mediated via the endothelium as pretreatment with deoxycholic acid (DCA) virtually abolished this response (Figure 4b). As the dilator response was not mediated via P_1 -purinoceptors (Figure 3), ATP may be acting via P_2 -purinoceptors on the endothelial layer.

ACh was found to have inconsistent effects on blood vessel calibre prior to elevation of vascular tone. In two preparations constriction was obtained, in one relaxation occurred and in a further three no effect was observed. However, when tone was elevated by perfusing with vasopressin (10^{-8} M), ACh then produced dose-dependent (0.2 to 20 nM) vasodilatation which was mediated via muscarinic receptors as it could be blocked by atropine. The receptors mediating this effect were located on the vascular endothelial layer as its removal virtually abolished the actions of ACh (Figure 6).

Figure 4 (a) Constrictor response to injected ATP before (■) and during perfusion (□) with the P_2 -purinoceptor desensitizer α, β methylene ATP (10^{-6} M). Responses in blood vessels prior to elevation of vascular tone ($n = 7$). (b) Dilator response to injected ATP before (■) and after removal of the endothelial layer (□) by sodium deoxycholate pretreatment. Vascular tone was elevated by perfusion with 5-hydroxytryptamine (10^{-5} M). $n = 6$ at each dose.

Figure 6 The dilator effect of acetylcholine (ACh) ($n = 9-12$) before (■) and after removal of the endothelial layer (□) by sodium deoxycholate pretreatment ($n = 6-8$). Vascular tone was raised by vasopressin (10^{-8} M).

Discussion

The purpose of the present study was twofold. Firstly, to establish whether purinoceptors are present in rabbit knee joint blood vessels and determine the effects they mediate. Secondly, to investigate whether any actions of purinoceptors are mediated via the endothelial layer of articular blood vessels.

Burnstock (1978) proposed that there were two subtypes of purinoceptors: P₁-purinoceptors, the interaction of which with adenosine can be antagonized by methylxanthines and P₂-purinoceptors which are more sensitive to ATP and can be antagonized by α,β methylene ATP (Burnstock, 1982; Kasakov & Burnstock, 1983). Research on various tissues in different species indicates that purine nucleotides act via P₁-purinoceptors to induce relaxation of vascular smooth muscle cells (Burnstock, 1982; Burnstock *et al.*, 1986) but that actions via P₂-purinoceptors produce contraction in some tissues and relaxation in others (Burnstock & Brown, 1981; Kennedy & Burnstock, 1985). The relaxation of smooth muscle cells can either be direct via receptors on smooth muscle cells or indirectly via receptors on the endothelial layer (Furchgott, 1981).

The results of the present experiments demonstrate the dual effect of ATP on articular blood vessels in the rabbit. ATP can produce vasoconstriction via P₂-purinoceptors on vascular smooth muscle. The desensitizing action of α,β methylene ATP which has been described previously (Kasakov & Burnstock, 1983; Hedlund *et al.*, 1983; Sneddon & Burnstock, 1984; Kennedy & Burnstock, 1985; Kennedy *et al.*, 1985) was confirmed here. Removal of the endothelium resulted in a slight increase in the vasoconstrictor action of ATP suggesting that the responses prior to DCA treatment were reduced by continuous release of endothelium derived relaxing factor (EDRF).

In contrast to ATP, adenosine did not produce any constrictor effect. However, after increasing the tone of joint blood vessels it caused concentration-dependent relaxation.

This response was mediated via P₁-purinoceptors on vascular smooth muscle as it was antagonized by 3-methylxanthine, but unaffected after removal of the endothelium. The weak effect of adenosine prior to elevation of vascular tone was probably due to this agent acting on blood vessels which were already vasodilated in the absence of tonic sympathetic constrictor discharge.

ACh was also found to exert a dilator effect which was consistently observed after increasing the vascular tone. However, this was virtually abolished by removal of the endothelium, which is similar to the results obtained by Furchgott & Zawadzki (1980) on rabbit thoracic aorta.

Contrary to an earlier report based on rabbit ear artery by Kennedy & Burnstock (1985), ATP appears to produce vasodilatation via P₂-purinoceptors, as 3-methylxanthine fails to block this effect whereas removal of the endothelium does. This finding is in agreement with another study on rat femoral artery carried out by Kennedy *et al.* (1985). Whether the dilatation is due to a direct action of ATP, or whether it is mediated by the breakdown products of ATP such as AMP and adenosine (Burnstock, 1978), remains to be answered. Previous reports have suggested that P₂-purinoceptors appear to play little or no role in ATP-elicited vasodilatation (Su, 1981; Kennedy & Burnstock, 1985). However, the results of the present experiments suggest that ATP acting presumably via P₂-purinoceptors on the endothelial cells of articular blood vessels does produce vasodilatation.

The physiological significance of the different distribution of P₁- and P₂-purinoceptors in different vascular beds remains unclear (Kennedy & Burnstock, 1985). However, it is likely that these purinoceptors and the endothelium may prove to have an important role in the regulation of flow through articular blood vessels.

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