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Receptors involved in nerve-mediated vasoconstriction in small arteries of the rat hepatic mesentery

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1 We have investigated the neurotransmitters and receptor subtypes involved in nerve-mediated vasoconstriction in small arteries of the rat hepatic mesentery.

2 A dense sympathetic innervation was demonstrated using catecholamine histochemistry and antibodies against the synaptic vesicle protein synaptophysin.

3 Reverse transcription-polymerase chain reaction (RT–PCR) demonstrated very strong expression of the α_{1A}-adrenergic, neuropeptide Y (NPY) Y_1, P_{2X1}- and P_{2X4}-purinergic receptors, moderate expression of the α_{2B}-adrenergic receptor and the purinergic P_{2X5}- and P_{2X7}-receptors and weak expression of the α_{1B}-, α_{1D}-, α_{2A}- and α_{2C}-adrenergic receptors and the P_{2X2}- and P_{2X3}-purinergic receptors. NPY_2 and P_{2X6} receptor expression was absent.

4 Electrical field stimulation (10 Hz, 10 s) produced contractions which were abolished by tetrodotoxin (10^{-6} M) and/or guanethidine (GE, 5×10^{-6} M) and a combination of benextramine (10^{-5} M) and α,β-methylene ATP, (α,β-mATP, 3×10^{-6} M) or PPADS (10^{-5} M). Selective α_1-adrenergic receptor antagonists showed the potency order of prazosin > WB-4101 > 5-methyl-urapidil > BMY 7378. Yohimbine (10^{-8} M, 10^{-7} M), α,β-mATP (3×10^{-6} M) and PPADS (10^{-5} M) each enhanced the response to nerve stimulation.

5 Some experiments demonstrated a slow neurogenic contraction which was abolished by GE or the selective NPY_1 receptor antagonist 1229U91 (6×10^{-7} M).

6 We conclude that nerve-mediated vasoconstriction results from the activation of postsynaptic α_{1A}-adrenergic and P_{2X}-purinergic receptors and under some conditions, NPY_1 receptors. Neurotransmitter release is modulated by presynaptic α_2-adrenergic receptors and possibly also P_{2X}-purinoceptors. The major postsynaptic subtypes involved were well predicted by mRNA expression as measured by RT–PCR, suggesting that this technique may be a useful adjunct to studies aimed at identifying functional receptor subtypes.

Keywords: α_{1A}-adrenoceptors; P_{2X}-purinoceptor; presynaptic receptors; neuropeptide Y_1 receptors; receptor mRNA expression

Introduction

Sympathetic nerves induce vasoconstriction in most vascular beds, however the mechanism by which they mediate this response is quite variable. This complexity arises from the fact that sympathetic nerves can release multiple transmitters, for example noradrenaline, ATP or neuropeptide Y (NPY) and these substances can act on a number of different pre- and postsynaptic receptor subtypes (Sneddon, 1995).

In general, sympathetic nerve-mediated contractions result from the activation of postsynaptic α-adrenoceptors and purinoceptors (Hirst *et al*., 1996). The specific α-adrenoceptors involved depend upon the vascular bed being studied. In the rat mesenteric arterial bed, the α_{1A}-adrenergic receptor is the predominant receptor involved in the response to nerve stimulation (Williams & Clarke, 1995), while in the rat iris vasoconstriction results from the activation of α_{1B}-adrenoceptors (Gould & Hill, 1994). In other vascular beds α_2-adrenoceptors can also participate in the vasoconstrictor response (Redfern *et al*., 1995). The purinergic component of nerve-mediated vasoconstrictions is mediated by P_{2X}-purinoceptors located on the vascular smooth muscle (Burnstock, 1995). The proportion of the response attributable to noradrenaline versus ATP depends upon the vascular bed, the species and the experimental stimulation parameters used (Ralevic & Burnstock, 1991).

Sympathetic nerve terminals possess presynaptic receptors that may either inhibit or enhance the release of the various transmitters (Langer & Arbilla, 1990). The nature of these presynaptic receptors will therefore affect the response seen in the postsynaptic tissue. The type of receptors involved in these presynaptic effects again vary between different vascular beds. In the guinea-pig saphenous artery, for example, presynaptic α_2-adrenergic and P_2-purinergic receptors inhibit noradrenaline release (Fujioka & Cheung, 1987), while in the rabbit mesenteric artery presynaptic α_2-adrenergic receptors inhibit noradrenaline and ATP release (von Kugelgen & Starke, 1985). Presynaptic receptors may also modify the release of other co-transmitters, for example the release of NPY from sympathetic nerve terminals can be inhibited by α_2-adrenoceptors (Dahlof *et al*., 1986).

In a previous study from this laboratory, we hypothesized that specific patterns of postsynaptic receptor gene expression might underlie some of the physiological differences seen between vascular beds (Phillips *et al*., 1997). Using reverse transcription-polymerase chain reaction (RT–PCR), it was shown that different vessels did indeed exhibit unique patterns of receptor mRNA expression. While these data for mRNA expression correlated well with data available from the functional studies of other investigators, a specific correlation of receptor mRNA expression and functional protein expression has not been made in the same tissue. Such a direct correlation would strengthen the suggestion that RT–PCR

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studies of receptor mRNA might be a useful adjunct to studies aimed at characterizing the specific postsynaptic receptors involved in neurogenic responses, particularly in situations of poor agonist and antagonist specificity and complications due to the activation of presynaptic receptors.

In this study we have used RT–PCR in conjunction with catecholamine histochemistry, immunohistochemistry and physiological techniques to investigate the specific identity of receptors involved in the constrictor response to nerve stimulation in small arteries of the rat hepatic mesentery bed. Previous studies of the main hepatic artery in several species have implicated α_1-adrenoceptors (Varga *et al*., 1984; Ballet, 1990; Ralevic *et al*., 1991) and P_{2X}-purinoceptors (Ralevic *et al*., 1991), although the specific receptor subtypes have not been identified nor has there been any evidence in favour of presynaptic mechanisms.

Methods

Experiments were performed on Wistar rats aged between 4 and 5 weeks postnatal. Animals were anaesthetized with ether anaesthetic and killed by cervical dislocation. For all experiments the hepatic artery proper was dissected from the point where it separated from the coeliac artery to the point where its branches enter the liver parenchyma.

Catecholamine histochemistry and immunohistochemistry

The mesentery containing the hepatic artery and its branches was stretched and pinned in a dish containing Sylgard (Dow Corning Corp. U.S.A.). For each histochemical technique, preparations from at least five animals were examined. For catecholamine histochemistry, whole mount preparations were fixed in 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, for 3 h at 4°C (Furness *et al*., 1977), dried over phosphorus pentoxide, mounted in paraffin oil and viewed with an Olympus BH microscope (Excitation filter BP 405, Dichroic mirror DM 455, Barrier filter 475).

For immunohistochemistry, whole mount preparations were fixed in Zamboni's fixative (2% (w/v) paraformaldehyde, 0.2% (w/v) picric acid in 0.1 M sodium phosphate buffer) for 1 h at 4°C. Picric acid was removed by treatment with dimethylsulphoxide (3×10 min) and the tissue washed and stored overnight in phosphate buffered saline (PBS) containing 0.04% (v/v) sodium azide. Whole mounts were then treated with 0.1 M glycine (3×10 min), washed in PBS containing 0.01% (v/v) Triton X and pre-incubated in PBS containing 0.2% (v/v) Triton X, 7 mg $ml^{-1}\lambda$ carrageenan and 0.01% (v/v) sodium azide for 1 h at room temperature. Primary antibody incubation with rabbit antibodies against human synaptophysin (1 : 30, DAKO Denmark) was for 48 h at room temperature. Synaptophysin labelling was detected after sequential incubation in biotinylated anti-rabbit secondary antibody IgG (1 : 650, VECTOR Labs. U.S.A.) and Texas Red conjugated to streptavidin (1 : 200, Amersham U.S.A.). Preparations were mounted in buffered glycerol and viewed with an Olympus BH microscope (Excitation filter BP 545, Dichroic mirror DM 580, Barrier filter R-610).

Receptor mRNA expression

RNA isolation Description of this procedure is given in full detail in an earlier publication (Phillips *et al*., 1997). Briefly, total cellular RNA was isolated from fresh tissue using a single step protocol for RNA extraction (RNAzol B′ Cinna Biotecx U.S.A.) after blood and connective tissue had been removed. Six to ten animals were used for each preparation and four such preparations were examined for mRNA expression. Genomic DNA was removed using Message Clean DNaseI (GenHunter Brookline U.S.A.) following the manufacturer's recommended protocol.

Reverse transcription-polymerase chain reaction To produce a complementary DNA (cDNA) pool, 5 μg of RNA was reverse transcribed using 200 U of reverse transcriptase enzyme (GIBCO Superscript II), 1 mM dNTP's (Pharmacia), 40 U of RNase inhibitor (Stratagene) and 300 ng of Random Primers (GIBCO) at 42°C for 58 min, 50°C for 58 min and then 90°C for 10 min to inactivate the enzyme. From the cDNA pool generated, 2 μl was used for each PCR reaction. The amount of product amplified was therefore relative to both a constant starting amount of RNA and cDNA. PCR was performed with subtype specific primers for the $\alpha_{1(A,B,D)}$- and $\alpha_{2(A,B,C)}$-adrenergic, NPY (Y_1, Y_2) and purinergic P_{2X} ($P_{2X1\text{-}7}$) receptor subtypes [see Alexander & Peters (1997) and Fredholm *et al*. (1997) for subtype classifications]. Primer sequences and PCR conditions have been published previously for the α-adrenergic and neuropeptide Y receptors (Phillips *et al*., 1997; Newhouse & Hill, 1997). Primers for the purinergic receptors were designed from published sequences (Collo *et al*., 1996; Surprenant *et al*., 1966) and are as follows-P_{2X1}; 5′-GTGTGCCCTTCAATGGCACTGTGA-3′, 5′-CCCATACAGTCCGTGGAACTGGTA-3′, P_{2X2}; 5′-CTGCCTCCTCAGGCTACAACTTCA-3′, 5′-GAGTACGCACCTTGTCGAACTTCT-3′, P_{2X3}; 5′-ATCAAGAACAGCATCCGTTTCCCT-3′. 5′-AGTGTTGTCTCAGTCACCTCCTCA-3′, P_{2X4}; 5′-GATCGTCACCGTGAACCAGACACA-3′, 5′-TCCACGATTGTGCCAAGACGGAAT-3′. P_{2X5}; 5′-TTTCTTCGTGGTCACCAACCTGAT-3′. 5′-ATTTGTGGAGCTGAAGTGACAGGT-3′, P_{2X6}; 5′-CAACTTCCTTGTGACACCAGCTCA-3′, 5′-GGAGAGTGAATCGTAGAGACAGTA-3′ and P_{2X7}; 5′-GTTTGACATCATCCAGTTGGTTGT-3′, 5′-ATCTTACTGAAGAGCTCAGAGGTA-3′. Annealing temperatures and extension times were 67°C and 40 s respectively for the P_{2X1}, P_{2X2} and P_{2X4} purinergic receptors, 65°C and 40 s for the P_{2X5} and P_{2X6} receptors, 65°C and 60 s for the P_{2X3} receptor and 63°C and 60 s for the P_{2X7} receptor. The PCR fragments were separated on 2% (w/v) agarose gels and visualized with ethidium bromide. The amount of product detected was subjectively assessed in comparison with a standard DNA ladder of known molecular weight and a scoring system of [0] to [+++] was used, where [0] = not detected, [+] = weakly detected, [++] = strongly detected, [+++] = very strongly detected.

In vitro *experiments*

After dissection, the hepatic arterial tree was immobilized by pinning the adjacent mesentery in a 1 ml bath whose base was covered in a thin layer of silicone (Sylgard, Dow Corning Corporation, Midland U.S.A.). Preparations were superfused with Krebs' solution (mM): NaCl 119.8, KCl 5.0, $CaCl_2.6H_2O$ 2.5, $MgCl_2.6H_2O$ 2.0, $NaH_2PO4.H_2O$ 1.0, $NaHCO_3$ 25 and D-glucose 27.7, gassed with 5% CO_2/95% O_2, at 34°C in the tissue bath. Hyoscine hydrochloride (10^{-6} M) and capsaicin (10^{-6} M) were added to the Krebs' solution at all times to prevent the effects of cholinergic and sensory nerves respectively. Preparations were allowed to equilibrate for 45 min prior to transmural stimulation (10 Hz, 10 s, 60 V, 0.1 msec pulse duration) every 20 min via platinum electrodes

placed 5 mm apart on opposite sides of the preparation. Preliminary experiments were performed to determine the minimal stimulation parameters, with regard to frequency and duration, that would produce a response of reasonable size that could be blocked by tetrodotoxin. The artery was visualized using video microscopy and the vessel diameter was continually monitored (DIAMTRAK, T.O. Nield 1996). Data were collected and measured on a Maclab Chart Recorder (ADInstruments U.S.A.). All experiments were performed on second or third order branches of the hepatic artery within the mesentery (mean resting diameter, 78.52 ± 2.23 μm, $n = 113$). These diameters would be larger than the actual vessel diameter *in vivo* due to the pinning of the surrounding mesentery, however vessels were not occluded and blood movement was always observed during nerve stimulation.

Control experiments were performed to determine the time period over which consistent responses could be achieved. The magnitude of the vasoconstrictor response to nerve stimulation was expressed as a percentage of the resting vessel diameter. This was done in order to standardize nerve-mediated responses in vessels of different resting diameter (Gould *et al*., 1995; Hill & Gould, 1995). Prior to the addition of any drug, the average of two to three nerve-mediated responses in control Krebs' was calculated. For each drug or specific drug concentration, at least two responses were averaged once a consistent response appeared. This meant that drugs were perfused for at least 20 min, the time between sequential nerve stimuli. The response in the drug solution was expressed as a percentage of the control response. Experimental values are given as the mean $\pm$ s.e.mean of results from at least four preparations, where each preparation was obtained from a different animal. All results were obtained with the appropriate drug present in solution except for the irreversible α-adrenergic blocker benextramine, whose effect was determined after a washout period of 20 min to avoid any non-specific actions. In experiments where the effect of a second drug was examined, the first drug remained present in solution for the duration of the experiment. When α,β-methylene ATP (α,β-mATP) was used to desensitize the P_{2X}-purinergic receptors, nerve-mediated responses were not measured until the vessel had recovered from the induced contraction.

Statistical significance was tested using a paired two tailed Students *t*-test and a P value of < 0.05 was taken as significant. Concentration response curves were constructed using Axograph (Axon Instruments) and a Hills χ^2 equation to fit the curves. The half maximal inhibitory concentration (IC_{50}) was calculated directly from the curves.

Drugs and solutions

The following drugs were used: tetrodotoxin, (−)hyoscine (scopolamine) hydrochloride, guanethidine sulphate (GE), benextramine tetrachloride, prazosin hydrochloride and yohimbine hydrochloride were all manufactured by SIGMA U.S.A. α,β-mATP, pyridoxal phosphate-6-azophenyl 2′-4′-disulphonic acid tetrasodium (PPADS), 5-methyl-urapidil, WB-4101 hydrochloride (WB4101) and BMY 7378 dihydrochloride (BMY7378) were all from Research Biochemicals Incorporated U.S.A. Capsaicin was purchased from Fluka Chemica (Switzerland) and 1229U91, an NPY_1 receptor antagonist (Lew *et al*., 1996), was kindly supplied by J. Angus and R. Murphy, University of Melbourne, Australia.

All drugs were made up as at least $1000 \times$ stocks in water except for capsaicin (100% ethanol), prazosin (20% v/v methanol) and 5-methyl-urapidil and WB4101 (0.1 M hydrochloric acid). Dilutions of all stocks were made in Krebs' for final concentrations. Diluents were tested at appropriate concentrations. Appropriate precautions were taken for light-sensitive drugs, including illuminating the preparations with only long wavelength light (> 610 nm).

Results

Catecholamine histochemistry and immunohistochemistry

Within the hepatic mesentery of the rat, the hepatic artery gives off multiple branches that supply the different lobes of the liver. A large nerve fibre bundle was seen to run parallel to the artery and its branches (Figure 1a,b). Catecholamine histochemistry revealed a dense perivascular plexus of catecholaminergic nerve fibres. The density of this plexus was consistent over the hepatic artery proper extending up to where

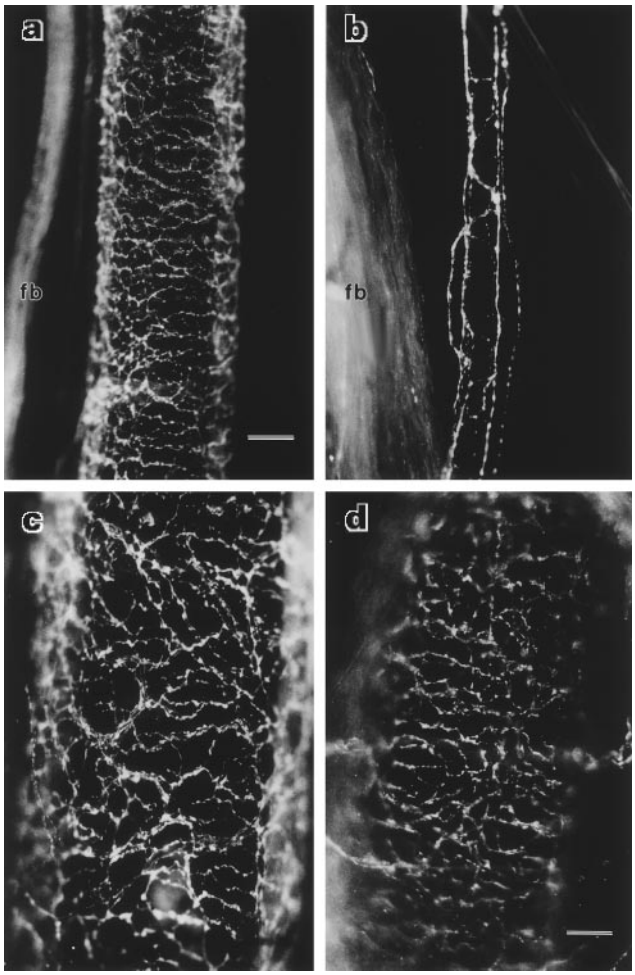

Figure 1 Catecholamine histochemistry and immunohistochemistry performed on arteries from the rat hepatic mesentery. For the catecholamine histochemistry (a, b and c) whole mount preparations were fixed in 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer. Immunohistochemistry (d) was performed with antibodies against synaptophysin to visualize synaptic vesicles. (a) Shows the the perivascular plexus of catecholaminergic fibres surrounding a second order vessel within the hepatic mesentery. (b) Shows an arteriole within the mesentery. In both (a) and (b) a nerve fibre bundle (fb) can be seen running parallel to the vessel. (c) Shows the catecholaminergic perivascular plexus of a secondary order vessel under higher magnification. (d) Shows the distribution of vesicles stained with antibodies against synaptophysin, detected with biotinylated anti-rabbit secondary antibodies and Texas Red conjugated to streptavidin. Scale bar represents 50 μm in (a) and 25 μm in (b, c and d).

the smaller order vessels entered the liver parenchyma (Figure 1a,c). Arterioles within the mesentery were also seen to have perivascular catecholaminergic nerve fibres associated with them although there were far fewer of them than over the larger vessels (Figure 1b). The measured vessel diameter obtained from photographic images was greater than that calculated from the *in vitro* experiments due to the greater stretch required to produce flat preparations and images of high resolution.

Antibodies directed against the synaptic vesicle protein synaptophysin revealed a similar distribution of varicosities over the surface of the arteries, however the intervaricose regions did not show the same intensity of staining as was seen for the catecholamine fluorescence (Figure 1d).

Receptor mRNA expression

Messenger RNA expression of the α_1- and α_2-adrenergic, NPY and P_{2X}-purinergic receptor subtypes in the arteries of the rat hepatic mesentery is presented in Table 1. All subtypes except the NPY_2 and the purinergic P_{2X6} receptors were detected (Figure 2, lane 9; Figure 3, lane 7). The α_{1A}-adrenergic, NPY_1 and P_{2X1}-, P_{2X4}-purinergic receptors showed very strong levels of detection (Figure 2, lanes 2 and 8; Figure 3, lanes 2 and 5). The α_{2B}-adrenergic receptor was strongly detected (Figure 2, lane 6) as were the purinergic P_{2X5} and P_{2X7} receptors (Figure 3, lanes 6 and 8) while the remaining receptors showed weak levels of expression (Figure 2, lanes 3, 4, 5 and 7; Figure 3, lanes 3 and 4). The relative levels of mRNA expression for the different receptors were consistent amongst the different mRNA preparations.

No predicted PCR product was seen when the reverse transcriptase enzyme was omitted from reactions or when RNA was omitted (Figure 2, lane 10; Figure 3, lane 9). The efficiency of the α-adrenergic and neuropeptide primer pairs has been confirmed in other tissues (Phillips *et al*., 1997; Newhouse & Hill, 1997) where an appropriately sized PCR product was detected at a level equivalent to [+ + +]. For the purinergic receptors, sensory ganglia were used as a positive control (Collo *et al*., 1996). All PCR fragments detected corresponded to the predicted fragment size for each receptor subtype. For the purinergic P_{2X1} receptor an extra band at approximately 320 bp was detected. This band was not seen in sensory ganglia or brain tissue, however it was also detected in

Table 1 Expression of mRNA for α_1-adrenergic, α_2-adrenergic, neuropeptide Y and purinergic receptors in arteries of the hepatic mesentery

Receptor subtype	*mRNA expression level*
α_1-adrenergic	
α_{1A}	+ + +
α_{1B}	+
α_{1D}	+
α_2-adrenergic	
α_{2A}	+
α_{2B}	+ +
α_{2C}	+
Neuropeptide Y	
Y_1	+ + +
Y_2	0
Purinergic	
$P_{2\times1}$	+ + +
$P_{2\times2}$	+
$P_{2\times3}$	+
$P_{2\times4}$	+ + +
$P_{2\times5}$	+ +
$P_{2\times6}$	0
$P_{2\times7}$	+ +

Crosses represent detection of PCR product which correspond to the size of the desired cDNA band for each set of subtype specific primers. The number of crosses reflects the amount of amplified product generated after 35 cycles. [0] = never detected; [+]weakly detected; [+ +] = strongly detected; [+ + +] = very strongly detected.

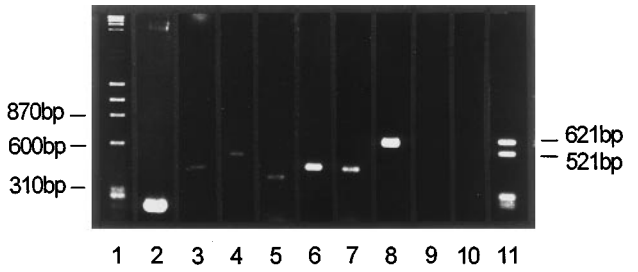

Figure 2 RT–PCR products generated using primers for rat $\alpha_{1(A,B,D)}$- and $\alpha_{2(A,B,C)}$-adrenergic and neuropeptide Y (Y_1, Y_2) receptors, from cDNA from the arteries of the rat hepatic mesentery. Figure illustrates agarose gel electrophoresis of 8 μl of PCR product, created after 35 cycles of amplification using subtype specific primers and cDNA that was reverse transcribed from DNaseI treated total RNA. A 251 base pair (bp) product in lane 2 corresponds to the α_{1A}-adrenergic receptor. Lanes 3 and 4 illustrate products of 405 and 517 bp corresponding to the predicted α_{1B}- and α_{1D}-adrenergic receptor fragments. Messenger RNA expression of the α_{2A}-, α_{2B}- and α_{2C}-adrenergic receptors is confirmed by the presence of bands of 338, 456 and 425 bp respectively in lanes 5, 6 and 7. Expression of mRNA for the neuropeptide Y Y_1 receptor was verified by the presence of a 589 bp product (lane 8) while no product was detected with the neuropeptide Y Y_2 primer pair (lane 9). No PCR product was seen in receptor specific control experiments that did not contain reverse transcriptase enzyme (not shown) or did not contain RNA (lane 10). DNA molecular weight markers (Lambda *Hind*III/ϕX174 *Hae*III) and (Ptz/Alu) appear in lanes 1 and 11 respectively.

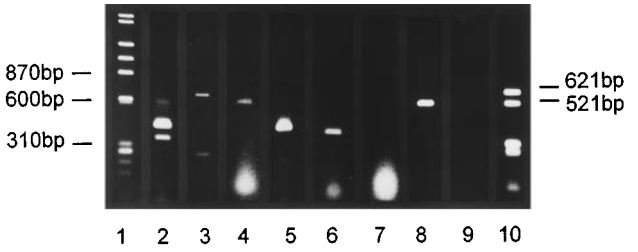

Figure 3 RT–PCR products generated using primers for the purinergic $P_{2X1\text{-}7}$ receptors, from cDNA from the arteries of the rat hepatic mesentery. Figure illustrates agarose gel electrophoresis of 8 μl of PCR product, created after 35 cycles of amplification using subtype specific primers and cDNA that was reverse transcribed from DNaseI treated total RNA. A 397 bp product in lane 2 corresponds to the P_{2X1}-purinergic receptor. An extra band at 320 bp is also present. Lane 3 contains a band at 273 bp corresponding to the predicted P_{2X2}-purinergic receptor. The band at 620 bp is a genomic DNA fragment created with the same primers. Lanes 4, 5 and 6 illustrate products of 575, 396 and 379 bp corresponding to the predicted P_{2X3}-, P_{2X4}- and P_{2X5}-purinergic receptor fragments. Messenger RNA expression for the P_{2X7}-purinergic receptor is confirmed by the presence of a band of 567 bp in lane 8. No product was detected with the P_{2X6}-purinergic receptor primer pair (lane 7). No PCR product was seen in receptor specific control experiments which did not contain RNA (lane 9). DNA molecular weight markers (Lambda*Hind*III/ϕX174*Hae*III) and (Ptz/Alu) appear in lanes 1 and 10 respectively.

RNA extracted from mesenteric artery. The band seen at 620 bp in Figure 3, lane 3 corresponds to amplification of genomic DNA by the P_{2X2}-purinergic primers and was seen in minus reverse transcriptase controls for this primer pair, in spite of the DNase treatment of the original RNA sample. Fragments corresponding to genomic DNA were not seen with other primer pairs.

In vitro *experiments*

General observations Preliminary studies indicated that a consistent contractile response could be achieved with stimulation of 10 Hz, 10 s (60 V, 1 ms pulse width) every 20 min. Whilst decreasing or increasing the stimulus frequency or duration caused a respective decrease or increase in the size of the contraction, shorter pulses and lower frequencies of stimulation failed to elicit consistent contractions of a size sufficient to analyse. Consequently all experiments were performed using 10 Hz for 10 s every 20 min. The average response to nerve stimulation was a contraction of $19.22 \pm 0.64\%$ ($n = 113$) of the resting vessel diameter. The time to peak contraction from the onset of the nerve stimulus was 10.49 ± 0.15 s ($n = 113$) and the time to 90% recovery of original resting vessel diameter was 35.19 ± 1.96 s ($n = 113$); (Figure 4a,b, middle panels). Preparations that showed a contractile response of less than 10% of resting vessel diameter were not used for further experiments. The contractile response was consistently blocked with TTX (10^{-6} M) and GE (5×10^{-6} M). Early experiments indicated that preparations could be stimulated with the above parameters for up to 5 h without a significant change in the magnitude of the nerve-mediated response ($n = 4$). When diluent controls were tested at the appropriate concentrations, they had no effect on the nerve-mediated contraction or resting vessel diameter. When α,β-mATP (3×10^{-6} M) was applied to preparations, either before or after treatment with any other drugs, an agonist induced contraction and subsequent desensitization occurred. This contraction, which was equal to $35.52 \pm 1.55\%$ ($n = 72$) of the resting vessel diameter, was a prolonged contraction and the vessel did not always fully recover. The average recovery was to $90.00 \pm 1.24\%$ ($n = 72$) of the resting vessel diameter within 20 min of the onset of the contraction.

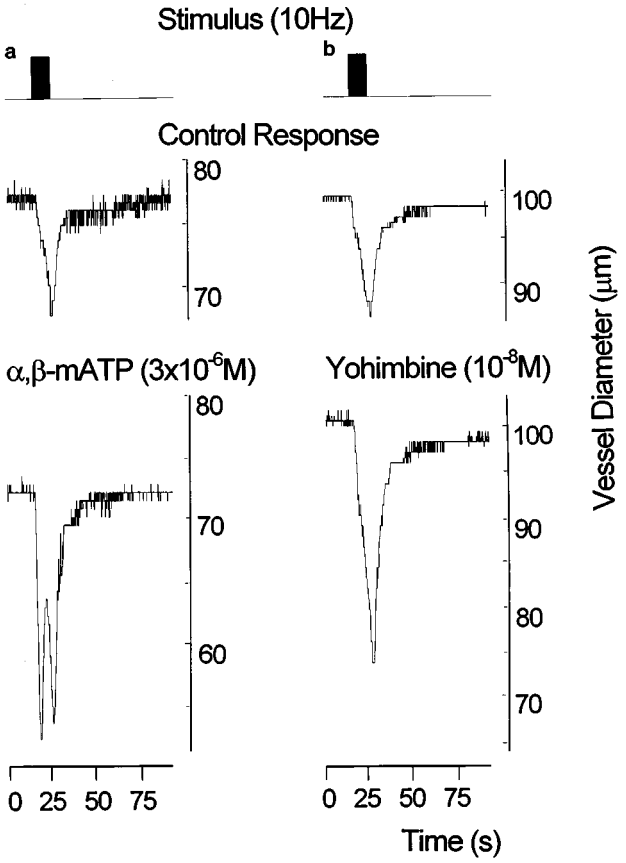

Figure 4 Representative traces showing the effect of (a) α,β-mATP (3×10^{-6} M) and (b) yohimbine (10^{-8} M) on the characteristics on the nerve-mediated contraction (μm) of arteries in the rat hepatic mesentery. Top panels show the nerve stimuli (10 Hz, 10 s, 60 V, 0.1 ms pulse width) which commenced 15 s after *time* = 0. Middle panels show the respective responses in Krebs' solution while the bottom panels illustrate the nerve-mediated response in the presence of (a) α,β-mATP or (b) yohimbine.

Components of contractile response To investigate what principal transmitters and receptors were involved in the contractile response to nerve stimulation, the effects of benextramine, $\alpha\beta$-mATP and PPADS were tested.

Benextramine (10^{-5} M) reduced the size of the nerve-mediated contraction to $49.23 \pm 4.19\%$ of control ($n = 15$); (data from Figure 5a and b combined). When α,β-mATP (3×10^{-6} M) was used after benextramine, the contractile response was further reduced to $5.77\% \pm 3.48\%$ of control ($n = 4$, Figure 5a). In those experiments where a residual component was present, it was removed after treatment with GE (5×10^{-6} M) and TTX (10^{-6} M, Figure 5a). When PPADS (10^{-5} M) was used after benextramine the response was similarly reduced (Figure 5b).

When the experiments described above were performed in the reverse order, with α,β-mATP or PPADS being used prior to benextramine, α,β-mATP or PPADS significantly enhanced the nerve-mediated contractile response (Figures 4a and 6a,b). The addition of benextramine (10^{-5} M) after either α,β-mATP or PPADS essentially abolished the enhanced response to nerve stimulation (Figures 6a,b).

In addition to the enhancement of the contractile response to nerve stimulation by both α,β-mATP and PPADS, the characteristics of the contraction were changed (Figure 4a). In the presence of α,β-mATP, the time to peak was significantly shorter than that in control Krebs' (5.88 ± 0.31 s, $n = 36$, $P < 0.05$) as was the recovery to 90% resting vessel diameter (25.22 ± 2.02 s, $n = 36$, $P < 0.05$). A similar phenomenon was seen in the presence of PPADS (time to peak: 4.54 ± 0.83 s, $n = 9$, $P < 0.05$, recovery: 30.97 ± 3.37, $n = 9$). In the majority of experiments, α,β-mATP (22/36) and PPADS (7/9) caused the nerve-mediated response to take on a biphasic appearance (Figure 4a). The addition of yohimbine (10^{-8} M) did not alter this biphasic time course ($n = 4$). A biphasic response was also seen in six control experiments. When this response was considered as two separate components, the first phase had a time to peak of 4.25 ± 0.17 s ($n = 35$), while the second phase had a time to peak of 10.87 ± 0.24 s ($n = 35$).

Identification of α-adrenoceptor subtypes In order to determine the specific postsynaptic receptor subtype responsible for the adrenergic component of the nerve-mediated constriction, a number of α-adrenoceptor antagonists were used.

The effects of different concentrations of the α_2-adrenoceptor antagonist yohimbine were tested (Figure 7). At no concentration did yohimbine cause a reduction in the amplitude of the contractile response although concentrations of 10^{-8} M and 10^{-7} M caused a significant enhancement of the size of the nerve-mediated contraction (Figures 4b and 7). This

potentiation was reversed at 10^{-6} M (Figure 7). Unlike α,β-mATP and PPADS, yohimbine did not produce a change in the time to peak contraction and a biphasic response was never seen (Figure 4b).

The responses to nerve stimulation in the presence of increasing concentrations of the α_1-adrenergic receptor antagonists BMY7378 (α_{1D}), 5-methyl-urapidil (α_{1A}), WB4101 ($\alpha_{1A/1D}$) and prazosin (α_1) were determined. The drugs showed the following potency order: prazosin > WB4101 = 5-methyl-urapidil > BMY7378 (Figure 8a), with responses being maximally inhibited by 82% for prazosin (10^{-8} M, 82.22±4.55%, $n=4$), by 63% for WB4101 (10^{-7} M, 63.09±8.86%, $n=4$) and by 60% for 5-methyl-urapidil (10^{-7} M, 59.94±7.31%, $n=4$). BMY7378 was virtually ineffective, only partially inhibiting the response at 10^{-7} and 10^{-6} M.

Experiments were also performed with the α_1-adrenergic receptor antagonists in the presence of α,β-mATP to eliminate the purinergic component of the contractile response and allow the curves to reach maximal inhibition. The contractile response in the presence of the specific α_1-adrenergic antagonists was expressed as a percentage of the α,β-mATP response in order for the control response to be equivalent to 100%. In these experiments, contractile response to nerve stimulation were extremely sensitive to prazosin ($IC_{50}=8\times10^{-10}$ M), WB4101 ($IC_{50}=1\times10^{-9}$ M) and 5-methyl-urapidil ($IC_{50}=2\times10^{-9}$ M), and nerve-mediated contractions were essentially abolished by the higher concentrations of these drugs (Figure 8b). BMY7378 was inactive and only partially reduced the response at non-specific concentrations (10^{-7} M, 10^{-6} M, Figure 8b).

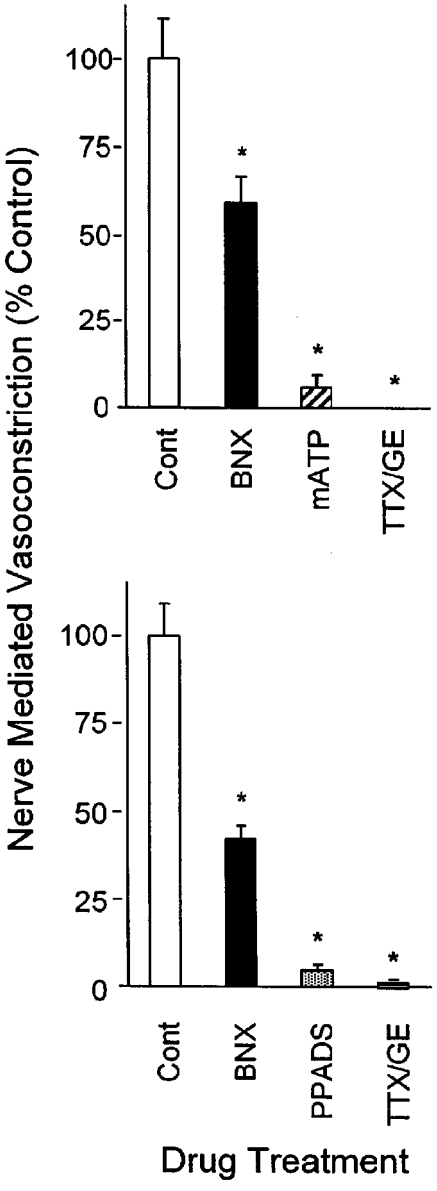

Figure 5 Effects of the cumulative consecutive application of (a) benextramine (BNX, 10^{-5} M) and α,β-mATP (mATP, 3×10^{-6} M) or (b) BNX and PPADS (10^{-5} M) on the nerve-mediated contractile response of arteries in the rat hepatic mesentery. Tetrodotoxin (TTX, 10^{-6} M) and/or guanethidine (GE, 5×10^{-6} M) abolished the small residual contraction. Columns represent the means±s.e.mean of at least 4 preparations. Results are expressed as % of the contractile response in control Krebs' solution (Cont). Control response in (a) was 20.78±2.64% of resting vessel diameter, $n=7$ and in (b) was 17.92±1.68%, $n=8$. *Differs significantly from control ($P<0.05$).

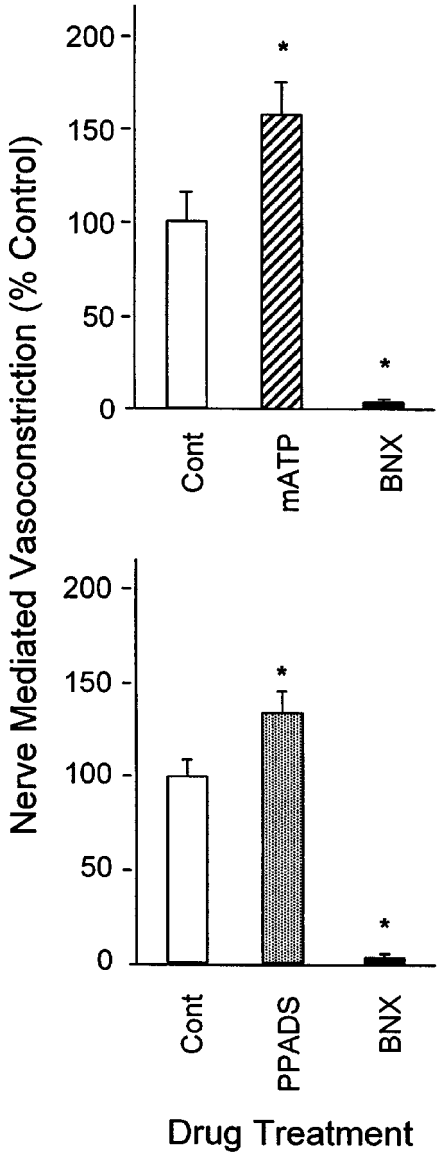

Figure 6 Effects of the consecutive application of (a) α,β-mATP (mATP, 3×10^{-6} M) and benextramine (BNX, 10^{-5} M) or (b) PPADS (10^{-5} M) and BNX on the nerve-mediated contractile responses of arteries in the rat hepatic mesentery. Columns represent the means±s.e.mean of at least six preparations. Results are expressed as % of the contractile response in control Krebs' solution (Cont). Control response in (a) was 22.10±3.62% of resting vessel diameter, $n=10$ and in (b) was 15.54±1.32%, $n=9$. *Differs significantly from control ($P<0.05$).

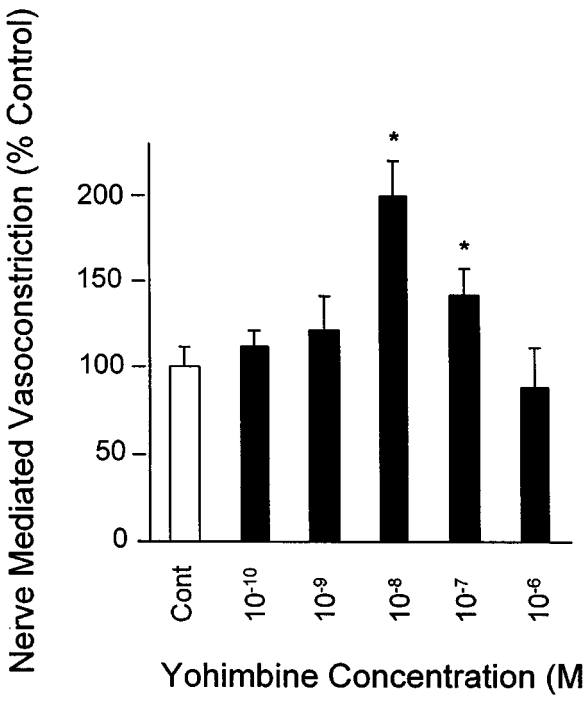

Figure 7 Effect of increasing concentrations of yohimbine on the nerve-mediated contractile response of arteries in the rat hepatic mesentery. Columns represent the mean±s.e.mean of at least 4 preparations. Results are expressed as % of the contractile response in control Krebs' solution (Cont), which was 19.51±2.31% of resting vessel diameter, $n=11$. *Differs significantly from control ($P<0.05$).

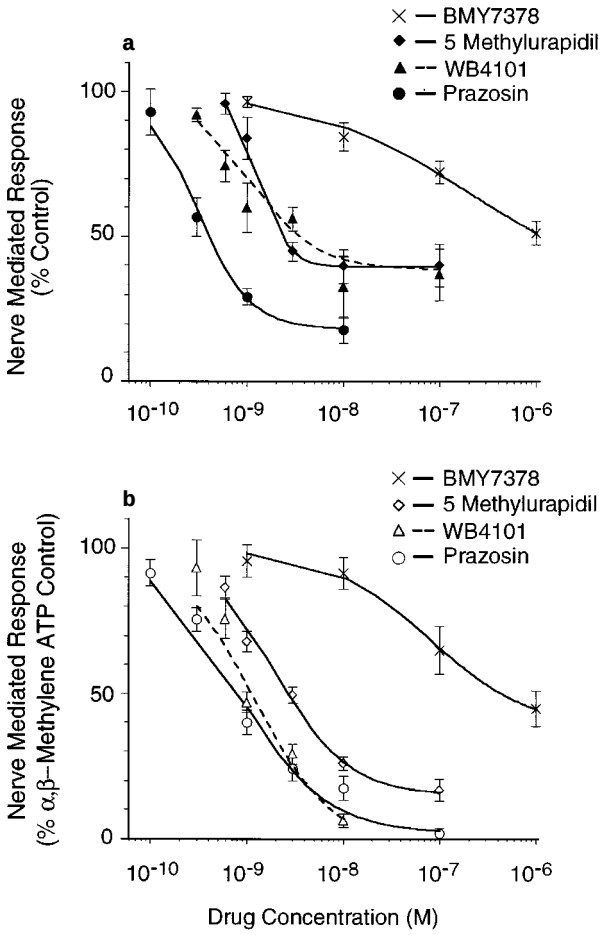

Figure 8 Contractile responses to nerve stimulation in the presence of increasing concentrations of α_1-adrenergic receptor antagonists, in arteries of the rat hepatic mesentery. (a) (full symbols) illustrates the effects of BMY7378 ($n=4$), 5-methyl-urapidil ($n=4$), WB4101 ($n=4$) and prazosin ($n=4$). (b) (open symbols) illustrates the effects of the same drugs after preincubation in the presence of α,β-mATP (3×10^{-6} M) (BMY7378: $n=4$, 5-methyl-urapidil: $n=4$, WB4101: $n=4$, prazosin: $n=4$). Results are expressed in (a) as % of the contractile response in control Krebs' solution (20.10±1.15% of resting vessel diameter, $n=29$) and in (b) as % of the contractile response after treatment with α,β-mATP (3×10^{-6} M, 22.41±1.21% of resting vessel diameter vs untreated control 15.90±1.11, $n=29$, $P<0.05$).

Nerve-mediated neuropeptide Y effects Treatment of the hepatic artery to remove the adrenergic and purinergic components of the contractile response uncovered a slow nerve-mediated contraction. The contraction had a time to peak amplitude of 41.57±2.46 s ($n=37$) and a time to recovery to 90% of resting vessel diameter of 110.34±8.75 s (Figure 9). The amplitude of the response was 10.73±1.03% ($n=36$) of the resting vessel diameter. This slow nerve-mediated contraction was blocked by TTX (10^{-6} M, $n=2$), GE (5×10^{-6} M, $n=10$) or 1229U91 (6×10^{-7} M, $n=7$). While the response was only seen once under control conditions, it appeared after treatment with the following drugs: benextramine ($n=20$), the P_{2X}-antagonists α,β-mATP and PPADS ($n=10$) either on their own or in combination with benextramine, and yohimbine ($n=6$). It should be noted however that the slow contraction was not always seen after the use of any of these drugs. 1229U91 was used to block the response in those experiments where a combination of benextramine and one of the purinergic receptor antagonists had uncovered the slow contraction.

Discussion

Transmural stimulation of the arteries of the rat hepatic mesentery at 10 Hz for 10 s, produced a response that was consistently blocked with tetrodotoxin and guanethidine, indicating that contractions were due to the release of neurotransmitter from sympathetic nerves and did not result from direct stimulation of the vascular smooth muscle (Hausler & Haefely, 1979). The principal transmitters involved in the contractile response under these stimulation conditions were noradrenaline and ATP, acting at α_1-adrenergic and P_{2X}-purinergic receptors respectively, as the response was effectively blocked by the combination of benextramine, an alpha-adrenoceptor antagonist (Benfey, 1982) and α,β-mATP, a stable analogue of ATP. The latter first activates and then desensitizes P_{2X}-purinergic receptors, and has therefore been used extensively as a P_{2X} antagonist (Kasakov & Burnstock, 1983; Burnstock & Kennedy, 1985). The result with α,β-mATP was confirmed using PPADS, a selective P_{2X}-purinoceptor antagonist (Ziganshin *et al*., 1994).

Catecholamine histochemistry revealed a dense plexus of catecholaminergic nerve fibres surrounding the walls of the arteries in the rat hepatic mesentery. The plexus continued over the major artery and its branches, extending to the points of entry of the vessels into the liver parenchyma. This is in agreement with an earlier fluorescence study by Ungvary & Donath (1969) who showed there was an abundant adrenergic

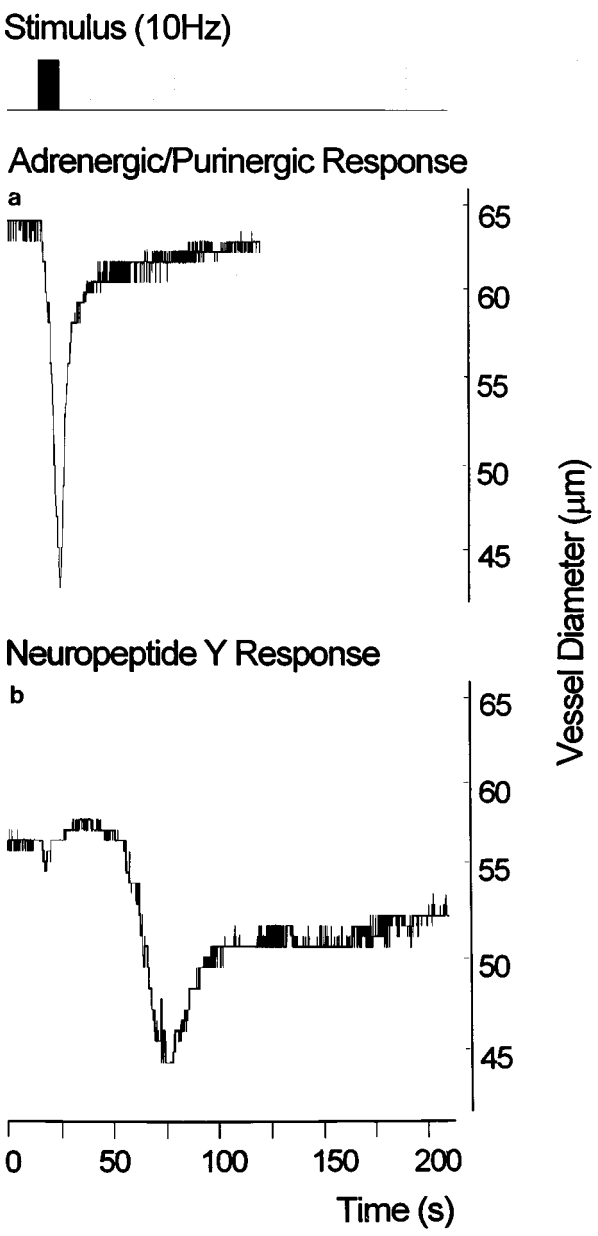

Figure 9 Representative traces showing the contractions due to (a) neurally released noradrenaline and ATP and (b) neurally released neuropeptide Y (μm). Top panel shows the nerve stimulus (10 Hz, 10 s, 60 V, 0.1 ms pulse width) which commenced 15 s after *time* = 0. The response in control Krebs' is shown in (a) and the neuropeptide Y response in the presence of α,β-mATP (3×10^{-6} M) and benextramine (10^{-5} M) is illustrated in (b).

nerve supply to the hepatic arteries of a number of different species, including the rat. Antibody staining with synaptophysin revealed a very similar pattern of staining, however the intervaricose regions were not stained to the same extent. This is to be expected as synaptophysin is an integral membrane component of synaptic vesicles (Wiedenmann & Franke, 1985) and has been implicated in the process of transmitter secretion (Alder *et al*., 1995). The similar punctate staining produced by catecholamine histochemistry and synaptophysin-like immunoreactivity confirms that these regions are the sites of neurotransmitter storage and release.

The proportion of the nerve-mediated contractile response attributable to noradrenaline depends upon both the vascular bed and the experimental conditions (Ralevic & Burnstock, 1991). Stimulation at low frequencies favours purinergic responses, while higher frequencies or longer periods of stimulation increase the relative component of the adrenergic response (Kennedy, 1996). In the arteries of the rat hepatic mesentery, stimulation parameters of 10 Hz for 10 s were required to produce a response of reasonable size that could still be blocked by tetrodotoxin. These results are in agreement with a study in the rabbit hepatic artery where contractions could not be induced at frequencies of less than 8 Hz (Brizzolara & Burnstock, 1990). Under these stimulation conditions of 10 Hz for 10 s, the response appeared to be primarily adrenergic with a smaller contribution from ATP acting on P_{2X}-purinoceptors, since the α_1-adrenergic antagonist, prazosin, blocked the majority of the response. On the other hand, the α-adrenoceptor antagonist, benextramine, only reduced the response by 50%, presumably due to increased release of ATP following blockade of presynaptic receptors as discussed below.

At present there are three different α_1-adrenergic receptor subtypes, α_{1A}, α_{1B} and α_{1D}, defined on the basis of their molecular and functional properties (Alexander & Peters, 1997). Pharmacological studies have suggested the subtypes α_{1L}, α_{1N} and α_{1H}, based on differential affinities for prazosin, with the presently known receptors classified as α_{1H} (Muramatsu *et al*., 1990). There is however no current molecular basis for the groups α_{1L} and α_{1N}. In the arteries of the rat hepatic mesentery, we characterized the subtype responsible for the α_1-adrenoceptor mediated component of the contraction, using a number of specific α_1-adrenoceptor antagonists. BMY7378, initially developed as a 5-hydroxytryptamine receptor antagonist (Yocca *et al*., 1987), has since been shown to be a selective antagonist of α_{1D}-adrenergic receptors (Piascik *et al*., 1995). In our preparation it did not affect the nerve-mediated contraction until high, non-specific concentrations were reached (Piascik *et al*., 1995). The hepatic artery preparation was however very sensitive to the selective α_{1A}-adrenergic receptor antagonists WB4101 and 5-methylurapidil (Hancock, 1996). These effects, combined with the apparent lack of effect of yohimbine, an α_2-adrenoceptor antagonist (Langer & Shepperson, 1982), strongly suggest that the contractile response to neurally released noradrenaline is mediated by α_{1A}-adrenergic receptors. These results correlated well with the strong mRNA expression for α_{1A}-adrenergic receptors as determined using RT–PCR.

RT–PCR studies demonstrated strong expression of mRNA for the P_{2X1}- and P_{2X4}-purinoceptors and moderate expression of the P_{2X5} and P_{2X7} receptors. The P_{2X1}-purinoceptor has previously been localized to vascular and visceral smooth muscle (Collo *et al*., 1996; Vulchanova *et al*., 1996). To our knowledge this is the first demonstration of P_{2X4}-, P_{2X5}- and P_{2X7}-purinergic receptor mRNA in a peripheral artery. Given the insensitivity of the of P_{2X4}, P_{2X5} and P_{2X7} receptors to α,β-mATP (Evans, 1996; Surprenant *et al*., 1996), it seems unlikely that these subtypes mediate the responses seen here. The receptor subtype mediating the purinergic component of the contraction is most likely the P_{2X1}-receptor.

It was interesting to find that yohimbine, α,β-mATP and PPADS all enhanced the size of the nerve-mediated contraction. Presynaptic modulation of transmitter release has been reported to be more significant at frequencies of stimulation > 8 Hz (Stjarne & Astrand, 1985; Shinozuka *et al*., 1990). The effect of yohimbine can be explained by the presence of presynaptic α_2-adrenergic receptors, on which noradrenaline acts to inhibit further transmitter release (Langer, 1974). By blocking these receptors, the negative feedback is removed and transmitter release is increased. Higher doses of yohimbine

(10^{-6} M) reversed the potentiation, possibly due to non-specific postsynaptic effects on α_1-adrenoceptors (Bao *et al*., 1993). The apparently greater purinergic component of the contractile response after benextramine, compared with prazosin, suggests that these prejunctional α_2-adrenoceptors also act to inhibit ATP release, as has been described in a number of different blood vessels (von Kugelgen & Starke, 1985; Bulloch & Starke, 1990; MacDonald *et al*., 1992).

The ability of α,β-mATP and PPADS to enhance the nerve-mediated contraction could also be attributed to their action in blocking presynaptic receptors. Treatment with α,β-mATP has been shown previously to enhance the release of noradrenaline from sympathetic nerves (Stjarne & Astrand, 1985; Shinozuka *et al*., 1990), through its action on presynaptic purinoceptors. The pharmacological sensitivity of the presynaptic purinergic receptor described here would suggest the involvement of a P_{2X}-purinoceptor. In addition to enhancing the amplitude of the nerve-mediated contraction, the P_{2X}-purinergic receptor antagonists also caused a reduction in the time to peak amplitude of the response and in some experiments the contraction becomes biphasic. Treatment with yohimbine also enhanced the nerve mediated contraction but did not produce any changes to the time course. The faster component is not due to the activation of extrasynaptic α_2-adrenoceptors (Langer & Shepperson, 1982) since yohimbine did not block the fast component of the response when added after a P_{2X}-purinoceptor antagonist. The change in time course may therefore be related to direct postsynaptic effects of the purinergic antagonists (Ralevic & Burnstock, 1990). Obviously, further work is required to clarify the mechanism by which α,β-mATP and PPADS could have this effect.

Another finding in this study on the arteries of the rat hepatic mesentery was the demonstration of a slow nerve-mediated contractile response in some experiments. This response possessed characteristics quite distinct from those of the contraction in control experiments, being much slower in the time to peak amplitude with a prolonged recovery time. Guanethidine and tetrodotoxin abolished the response, indicating that it was due to transmitter release from sympathetic nerves. Based on RT–PCR results, which indicated strong expression of mRNA for the NPY_1 receptor, we applied the selective NPY_1 receptor antagonist 1229U91 (Lew *et al*., 1996) and successfully blocked the response. In blood vessels, neurally released NPY usually mediates contractions via NPY_1 receptors (Grundemar *et al*., 1992) and its effects are slower to develop and more long lasting than those of its co-transmitters, noradrenaline and ATP (Morris *et al*., 1995).

In the present study NPY release was seen most frequently following the application of benextramine. It is likely that by blocking presynaptic α_2-adrenergic receptors, benextramine removed a noradrenaline mediated inhibition of NPY release. Inhibition of NPY release by presynaptic α_2-adrenergic receptors has previously been described (Dahlof *et al*., 1986). Yohimbine was also seen to induce the release of NPY, further supporting this hypothesis. The ability of α,β-mATP and PPADS to uncover the NPY response indicates a role for presynaptic inhibition of NPY release by ATP.

This study has shown that the arteries of the rat hepatic mesentery are subject to complex control mechanisms involving a number of different presynaptic and postsynaptic receptor subtypes. The major postsynaptic subtypes involved were well predicted by mRNA expression as measured by RT–PCR, suggesting that this may be a very useful adjunct to studies aimed at identifying functional receptor subtypes. Further elucidation of the dominant control mechanisms operating in the hepatic artery in pathological conditions, for example cirrhosis, may lead to the development of specific therapies to enhance or restrict blood flow as required.

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