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COMPARATIVE PHARMACOLOGICAL AND HISTOCHEMICAL EVIDENCE FOR PURINERGIC INHIBITORY INNERVATION OF THE PORTAL VEIN OF THE RABBIT, BUT NOT GUINEA-PIG

GEOFFREY BURNSTOCK, RAHIMA CROWE & HELEN K. WONG

Department of Anatomy & Embryology, University College London, Gower Street, London WC1E 6BT

1 Intramural nerve stimulation elicited a powerful relaxation of the longitudinal muscle of the rabbit portal vein in the presence of atropine and guanethidine, but not of the guinea-pig portal vein.

2 Intramural nerve stimulation of the rabbit portal vein produced a 13 fold increase in release of ^{3}H-adenyl compounds after preloading with [^{3}H]-adenosine. About 50% of this release was abolished by guanethidine. All release was abolished by tetrodotoxin. No significant release of radioactive compounds was observed during intramural nerve stimulation of the guinea-pig portal vein in the presence of guanethidine, although there was a 6 fold increase in release of radioactivity in the absence of drugs.

3 Histochemical studies using quinacrine, which binds ATP showed a fine fluorescent nerve plexus, nerve bundles, and ganglion cells in the rabbit portal vein, but not in the guinea-pig portal vein. This plexus was still present after chemical sympathectomy with 6-hydroxydopamine.

4 Adenosine 5′-triphosphate (ATP) relaxed the rabbit portal vein, but usually produced a biphasic response, consisting of a contraction followed by a relaxation, of the guinea-pig portal vein.

5 Prostaglandins E_1 and E_2 caused contraction of the rabbit portal vein. Indomethacin, a prostaglandin synthesis inhibitor, potentiated the relaxations of the rabbit portal vein produced by both non-adrenergic, non-cholinergic nerve stimulation and ATP.

6 High concentrations of antazoline and phentolamine, which antagonize purinergic responses in the guinea-pig taenia coli, caused a loss of basal tone so that it was not possible to assess their effects on the responses of the portal vein to either non-adrenergic, non-cholinergic nerve stimulation, or ATP.

7 Comparison of the results on the portal vein of the rabbit and guinea-pig provides support for the view that: (i) quinacrine fluorescence can be used to localize purinergic nerves and that the rabbit portal vein is supplied by these nerves; (ii) ATP is released from adrenergic nerve fibres, although, based on histochemical analysis, about 3 to 7 times less than is released from purinergic nerve fibres.

Introduction

The widespread occurrence of non-adrenergic, non-cholinergic nerves in the autonomic nervous system of vertebrates is well recognised (Burnstock, 1969; 1975a; Campbell, 1970). Evidence has been presented that a purine nucleotide, probably adenosine 5′-triphosphate (ATP) is the neurotransmitter in some of these nerves (Burnstock, Campbell, Satchell & Smythe, 1970; Burnstock, 1972; 1975b) and they have therefore been termed 'purinergic' (Burnstock, 1971).

The rabbit portal vein has a non-adrenergic, non-cholinergic innervation and ATP has been considered as a possible transmitter in these nerves (Hughes & Vane, 1967; 1970; Su, 1975). In the present experiments, we have examined the effect of various pharmacological treatments known to modify responses to purinergic nerve stimulation and ATP in other tissues (Satchell, Lynch, Bourke & Burnstock, 1972; Satchell, Burnstock & Dann, 1973; Burnstock, Cocks, Paddle & Staczewska-Barzcak, 1975) on the non-adrenergic, non-cholinergic, inhibitory response of the rabbit portal vein to intramural nerve stimulation. In addition, measurements have been made of the release of tritiated nucleotides from rabbit portal veins preloaded with [^{3}H]-adenosine during nerve stimulation.

The fluorescent compound quinacrine, an antimalarial acridine known to bind to ATP (Irvin & Irvin, 1954), has been used to demonstrate histochemically, fluorescent nerve cell bodies and fibres in

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the mammalian gastrointestinal tract and urinary bladder, both of which are claimed to contain intramural purinergic neurones (Olson, Ålund & Norberg, 1976; Burnstock, Cocks, Crowe & Kasakov, 1978a). We have now applied this method to the rabbit portal vein.

Since preliminary studies did not reveal non-adrenergic, non-cholinergic nerves in the guinea-pig portal vein, a comparative study of the pharmacology, release of tritiated adenyl compounds and fluorescence histochemistry has been extended to this species.

Methods

Pharmacological preparations

Rabbits of either sex, 1 to 2 kg in body weight, were killed by cervical dislocation. A 2 cm length of the portal vein was removed as described by Hughes & Vane (1967). The veins were cut open longitudinally, then suspended vertically in 20 ml organ baths under 3 to 4 g tension, since they have been found to give optimal responses under these conditions (Hughes & Vane, 1967). The baths were filled with Krebs physiological solution (Bülbring, 1953) at 38°C and bubbled with 95% O_2 and 5% CO_2. The tissues were allowed to equilibrate for 1 to 2 hours. Isometric tension of the longitudinal muscle of the vein was recorded with a Dynamometer UFI strain gauge, recording on a Grass 79D polygraph. Intramural nerve stimulation was with square wave pulses of 0.2 to 0.3 ms duration and supramaximal voltage, delivered via platinum ring electrodes 2 mm apart connected to a Grass S44 stimulator. Chemical sympathectomy was achieved by two intraperitoneal injections of 6-hydroxydopamine, separated by 48 h (total dose 250 mg/kg), the second injection being given 2 days before the animal was used (Malmfors & Thoenen, 1971).

Guinea-pigs of either sex (500 to 600 g) were killed by cervical dislocation and exsanguinated. The portal vein was removed and suspended vertically under 1 g tension and the experiments carried out under the same conditions as described above.

Radioactivity studies

For studies with [^{3}H]-adenosine the rabbit portal veins were incubated for 1 h in 3 ml of Krebs physiological solution in a jacketed organ bath at 37°C with [^{3}H]-adenosine (4×10^{-7} M, sp. act. 24 Ci/mmol). The incubation solution was gassed with a mixture of 5% CO_2 in O_2. At the end of the incubation period, the tissues were washed briefly in adenosine-free Krebs solution and mounted vertically under 1 to 3 g tension. They were then superfused at 3 ml/min with adenosine-free Krebs solution for 1.5 h. After the wash period, the veins were stimulated electrically at a frequency of 30 Hz, 0.3 ms pulse duration, at supramaximal voltage for 1 min.

For each stimulation, 10 successive 30 s fractions of superfusate were collected, consisting of 2 pre-stimulation samples and the rest during and after stimulation. The same procedure was repeated for the guinea-pig portal vein. For the radioactivity counting, a 1 ml aliquot of each fraction was mixed with 10 ml of liquid scintillation solution containing 55 mg of 2-5-diphenyloxazole (PPO), 0.5 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 3 ml of Triton-X 100 and the balance toluene. The samples were each counted for 10 min (giving total counts of approximately 500 counts/min) in an ABAC SL40 liquid scintillation counter.

Histochemical methods

Quinacrine fluorescent histochemistry The portal veins were dissected and washed in modified Krebs solution (Bülbring, 1953) at 0°C. The tissues were then incubated in Krebs solution containing quinacrine dihydrochloride (5×10^{-7} M) for 1 h at 37°C and gassed with 95% O_2 and 5% CO_2. Whole mounts were air-dried and mounted in liquid paraffin.

For tissue sections, the portal veins were freeze-dried at 30°C under 2×10^{-2} mmHg vacuum pressure in a Haak freeze-drier for 4 days. They were wax embedded under vacuum for 45 min. Transverse sections, 10 μm thick, were cut and mounted in liquid paraffin. All the tissues were viewed with a Zeiss photomicroscope fitted with an Epi-fluorescence condenser III R.S. Selected areas were photographed on Ilford HP5 film.

Catecholamine fluorescent histochemistry Portal veins were slit open longitudinally, stretched on microscope slides and air-dried over phosphorous pentoxide for 1 h and then treated with gaseous paraformaldehyde according to the Falck-Hillarp technique (Falck, 1962; Falck, Hillarp, Thieme & Torp, 1962). The stretch preparations (and also tissue sections) were mounted, viewed and photographed according to the methods given above.

Cholinesterase histochemistry Whole mounts of the portal vein were stained for acetylcholinesterase according to the method described by Karnovsky & Roots (1964). The tissues were dehydrated, cleared and mounted in Gurr's DePex. Selected areas were viewed through a Zeiss photomicroscope.

Drugs

The following drugs were used: ascorbic acid (BDH); adenosine (Sigma); [^{3}H]-adenosine (Amersham

Radiochemicals Ltd); adenosine triphosphate disodium salt (Sigma); antazoline hydrochloride (CIBA); atropine sulphate (Antigen Ltd); dipyridamole (Boehringer); guanethidine sulphate (CIBA); histamine dihydrochloride (Sigma); 6-hydroxydopamine hydrochloride (Sigma); indomethacin (Sigma); phenoxybenzamine (Smith, Kline & French Ltd); phentolamine mesylate (CIBA); prostaglandins E_1 and E_2 (Dr J. Pike, Upjohn, Kalamazoo, Michigan, U.S.A.); quinacrine dihydrochloride (Sigma); tetrodotoxin (Sigma). All drugs were dissolved in Krebs physiological solution. The 6-hydroxydopamine was dissolved in sterile saline solution (0.9% w/v NaCl solution) containing 0.2% w/v ascorbic acid.

Results

Rabbit portal vein

The basal tone of the tissue was assessed from the response to stimulation: preparations with low basal tone gave large contractile responses and small relaxations; those with high basal tone gave small contractile responses and large relaxations. In most experiments the basal tone remained at the level set initially; in approximately 20% of experiments there was a gradual increase of 0.5 to 2 g tension during the first hour. The rabbit portal vein usually exhibited spontaneous activity, although the fluctuations were small compared with the amplitude of the nerve-mediated responses.

Under the conditions used (see methods) the basal tone was usually low, so that in order to demonstrate responses to inhibitory nerves, it was necessary to raise the tone with ergotamine.

Intramural nerve stimulation and noradrenaline Intramural nerve stimulation (0.5 to 40 Hz at 0.3 ms pulse duration and supramaximal voltage for 10 s) elicited a contraction followed by an after-relaxation (Figure 1) or by an inhibition of spontaneous activity. In most preparations, maximal responses to intramural nerve stimulation were obtained at a frequency of 20 to 30 Hz (Figure 1). In a few preparations with especially high basal tone intramural nerve stimulation elicited only relaxation.

Noradrenaline (6×10^{-8} to 3×10^{-5} M) produced dose-dependent contractions, sometimes followed by inhibition of spontaneous activity.

Intramural nerve stimulation in the presence of adrenoceptor antagonists, adrenergic neurone blocking agents and atropine

Adrenoceptor antagonists The contractions of the portal vein to intramural nerve stimulation or noradrenaline were abolished by the α-adrenoceptor antagonists, phentolamine (5×10^{-6} to 10^{-5} M) and

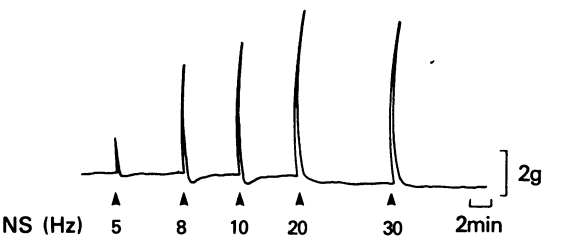

Figure 1 Isometric contractile responses of the rabbit portal vein to intramural nerve stimulation (NS) at various frequencies (Hz), 0.3 ms pulse duration, and supramaximal voltage, for 10 s periods.

phenoxybenzamine (10^{-5} M), neither of which altered the tone of the preparation. After α-adrenoceptor blockade, the response to intramural nerve stimulation was reversed to a relaxation, which was only slightly reduced by β-adrenoceptor blockade with propranolol (10^{-8} to 5×10^{-7} M). In the presence of α-adrenoceptor antagonists, noradrenaline gave a small residual relaxation which was blocked by propranolol (10^{-8} to 5×10^{-7} M). The inhibitory response was not affected by atropine (2×10^{-7} to 5×10^{-7} M) or guanethidine (Figure 2b).

Ergotamine (8.6×10^{-6} M) as well as being an α-adrenoceptor antagonist, increased the tone of the vein, which enhanced the relaxation responses; it was therefore used routinely for studies of inhibitory responses (Figure 2a). During the transition from adrenergic excitation to non-adrenergic relaxation in the presence of ergotamine, the response was complex, consisting of a small fast initial relaxation, followed by a fast contraction, before the main inhibitory response occurred (Figure 2c).

Guanethidine In the presence of the adrenergic neurone blocker, guanethidine (2×10^{-7} to 2×10^{-6} M), intramural nerve stimulation of the portal vein elicited a relaxation. In the high tone preparation illustrated in Figure 3, the relaxation was fast in onset and reached a maximum at a frequency of stimulation of about 25 to 35 Hz. At these concentrations, guanethidine did not affect the basal tone, regardless of whether it was high or low.

Responses to ATP and adenosine Exogenous ATP (10^{-6} to 10^{-3} M) produced dose-dependent relaxations of the rabbit portal vein, which were enhanced in the presence of ergotamine. In 40% of the preparations, ATP elicited a fast relaxation followed by a slow one (Figure 4a) and in 20% of the preparations, a relaxation followed by a contraction (Figure 4b). In the remaining preparations, ATP elicited only a slow relaxation (Figure 4c). The slow relaxation had latent periods of 45 to 75 s and reached a maximum magnitude after 1.5 to 2 min. The latent period de-

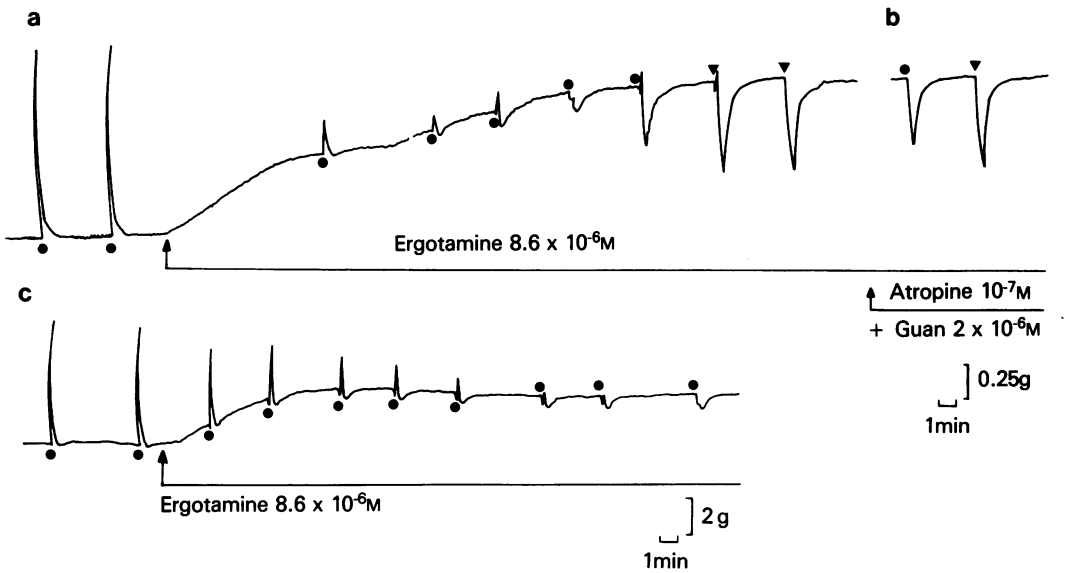

Figure 2 (a) Reversal of isometric responses of the rabbit portal vein to intramural nerve stimulation, at frequencies of 10 Hz (●) and 15 Hz (▼), from contractions to relaxations by the administration of the α-adrenoceptor antagonist ergotamine (8.6×10^{-6} M). (b) The relaxations are unaffected by guanethidine (Guan, 2×10^{-6} M) and atropine (10^{-7} M). (c) In a second preparation, in the presence of ergotamine (8.6×10^{-6} M), the response to intramural nerve stimulation (●, at a frequency of 7 Hz) is gradually reversed from a contraction to a relaxation. During the transition phase, the response to intramural nerve stimulation is triphasic, consisting of a small fast relaxation, followed by a contraction and then a secondary slow relaxation.

Figure 3 Non-adrenergic, non-cholinergic relaxations of the rabbit portal vein to intramural nerve stimulation (NS, ●, at various frequencies (Hz), for 10 s periods). Guanethidine (2×10^{-6} M), ergotamine (8.6×10^{-6} M) and atropine (2×10^{-7} M) present.

creased and the rate of relaxation increased as the ATP dose was increased. Adenosine (5×10^{-6} to 10^{-3} M) was less potent than ATP in relaxing the portal vein.

Effect of drugs on the responses to non-adrenergic nerve stimulation and ATP

Tetrodotoxin Tetrodotoxin (1.6×10^{-6} to 1.6×10^{-5} M), which blocks nerve propagation but not smooth muscle activity (Kao, 1966), abolished the non-adrenergic relaxations of the portal vein to intramural nerve stimulation without affecting the basal tone of the preparation (Figure 5).

Chemical sympathectomy with 6-hydroxydopamine Chemical sympathectomy with 6-hydroxydopamine abolished the responses to intramural nerve stimulation and revealed inhibitory responses which were comparable to those seen with phentolamine and phenoxybenzamine.

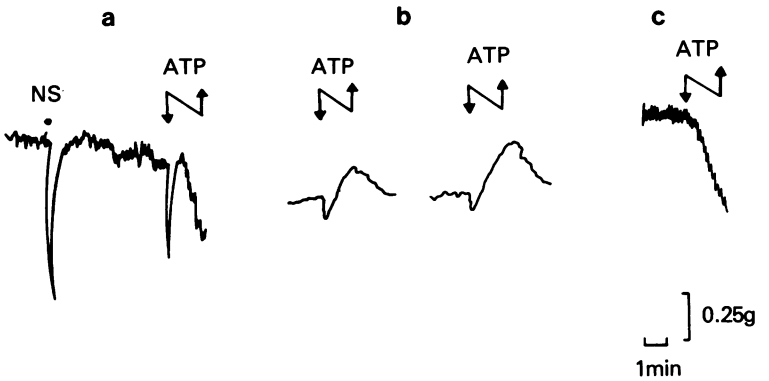

Figure 4 Isometric responses of the rabbit portal vein to intramural nerve stimulation (NS, at a frequency of 7 Hz) and ATP. The responses to ATP are of 3 types: (a) a fast relaxation followed by a secondary slow relaxation (ATP 5×10^{-4} M); (b) a slow relaxation followed by a contraction (ATP 10^{-4} M and 5×10^{-4} M) and (c) a slow relaxation only (ATP 5×10^{-4} M). Guanethidine (2×10^{-6} M) and ergotamine (8.6×10^{-6} M) present.

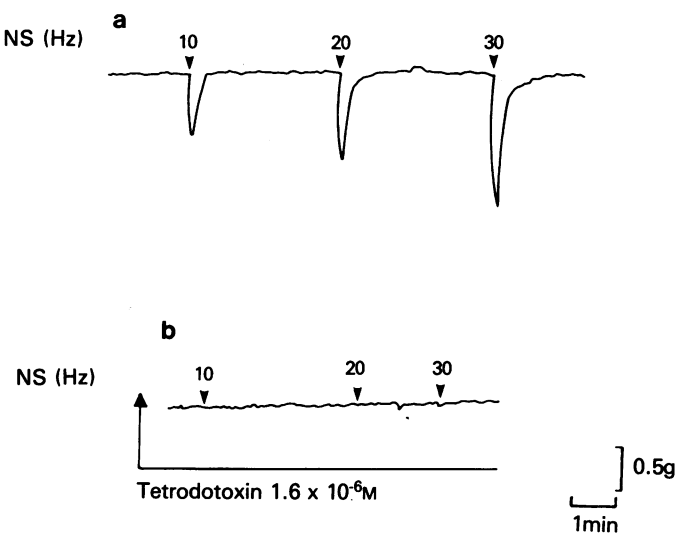

Figure 5 Responses of the rabbit portal vein to intramural nerve stimulation (NS, ▼, at various frequencies (Hz) for 10 s periods) before (a) and (b) 30 min after the administration of tetrodotoxin (1.6×10^{-6} M). Guanethidine (2×10^{-6} M) and ergotamine (8.6×10^{-6} M) present.

Indomethacin The prostaglandin synthesis inhibitor, indomethacin, has been shown to potentiate the relaxation to purinergic nerve stimulation and ATP and to block the 'rebound contraction' following cessation of stimulation in the guinea-pig taenia coli (Burnstock *et al.*, 1975).

In the rabbit portal vein, indomethacin, at concentrations of 5×10^{-6} M to 10^{-5} M, was found to potentiate the responses to both non-adrenergic, non-cholinergic nerve stimulation and ATP without affecting the basal tone of the preparation (Figure 6).

Prostaglandins E_1 and E_2 (1.7×10^{-7} to 1.7×10^{-6} M), produced a contraction of the vein which was sustained for up to 30 min after washout.

Dipyridamole Dipyridamole (10^{-7} to 10^{-6} M), an adenosine uptake inhibitor, has been reported to potentiate the responses to both ATP and purinergic

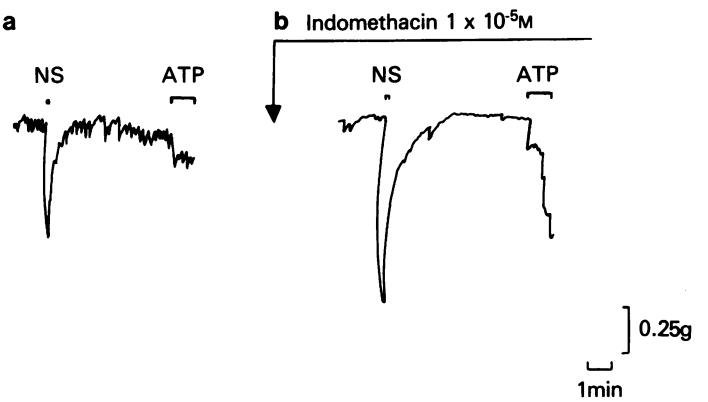

Figure 6 Responses of the rabbit portal vein to intramural nerve stimulation (NS, 10 Hz, for 10 s) and ATP (5×10^{-5} M) (a) before and (b) in the presence of indomethacin (1×10^{-5} M) for 1 h. Guanethidine (2×10^{-6} M) and ergotamine (8.6×10^{-6} M) were present throughout.

nerve stimulation in the guinea-pig taenia coli (Satchell *et al.*, 1972). In the rabbit portal vein, dipyridamole (5×10^{-7} to 10^{-6} M) produced a loss of tone and did not increase the relaxation in response to non-adrenergic intramural nerve stimulation and ATP.

2-substituted imidazolines High concentrations (1.8×10^{-4} M) of the 2-substituted imidazolines, phentolamine and antazoline, inhibited the relaxation in response to purinergic nerve stimulation in the guinea-pig taenia coli (Satchell *et al.*, 1973). As with the taenia coli, antazoline and phentolamine in concentrations which produced no change in tone (up to 1×10^{-5} M) had no effect on the non-adrenergic relaxation or ATP in the rabbit portal vein. Higher concentrations of these drugs (3.5×10^{-5} to 1.8×10^{-4} M) caused a loss of tone so that it was not possible to assess their effects on the responses to non-adrenergic nerve stimulation or ATP.

Release of ^{3}H-adenyl compounds It has been shown that [^{3}H]-adenosine is taken up by the rabbit portal vein, transformed into ^{3}H-nucleotides, mainly [^{3}H]-ATP, and stored in this form (Su, 1975). Thus it was of interest to examine the release of ^{3}H-adenyl compounds before and after treatment of the portal vein with guanethidine and tetrodotoxin.

After incubation of the veins in [^{3}H]-adenosine, intramural nerve stimulation (at 30 Hz, 0.3 ms, and supramaximal voltage for 60 s) in the absence of drugs, caused a 13 fold (13.0 ± 2.8; mean ± s.e., $n = 4$) increase in ^{3}H-adenyl compounds above prestimulation levels. In veins that were treated with guanethidine (6×10^{-6} M), intramural nerve stimulation (30 Hz, 0.3 ms, supramaximal voltage for 60 s)

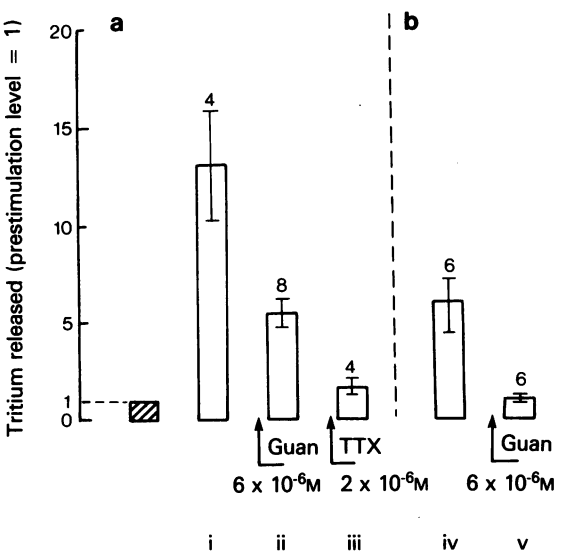

Figure 7 (a) Rabbit: release of tritium from the portal vein during intramural nerve stimulation (30 Hz for 60 s). (i) in the absence of drugs, (ii) in veins treated with guanethidine (Guan, 6×10^{-6} M) and (iii) in veins treated with tetrodotoxin (TTX, 2×10^{-6} M). (b) Guinea-pig: release of tritium from the portal vein by intramural nerve stimulation (NS, 30 Hz for 60 s), (iv) in the absence of drugs, and (v) in veins treated with guanethidine. In all experiments, drugs were present for at least 30 min before intramural nerve stimulation, and remained present throughout the experiment. Amount of tritium released is expressed as increase above a prestimulation level of unity (hatched column). The number of experiments performed is shown inside the columns. Vertical bars denote standard errors.

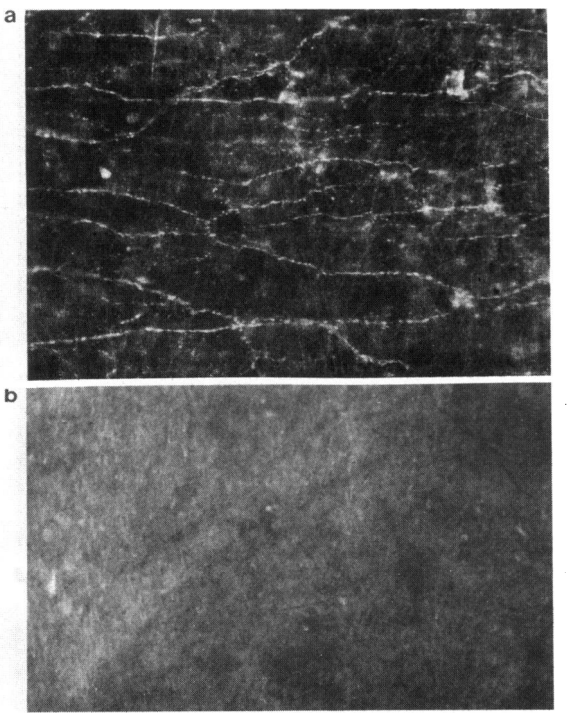

Figure 8 Quinacrine fluorescence histochemistry on rabbit and guinea-pig portal veins. (a) Rabbit portal vein. A plexus of fine varicose fibres is seen. The background fluorescence is very low. Fluorescent micrograph × 106. (b) Guinea-pig portal vein. No quinacrine-positive elements are seen. Fluorescent micrograph × 108.

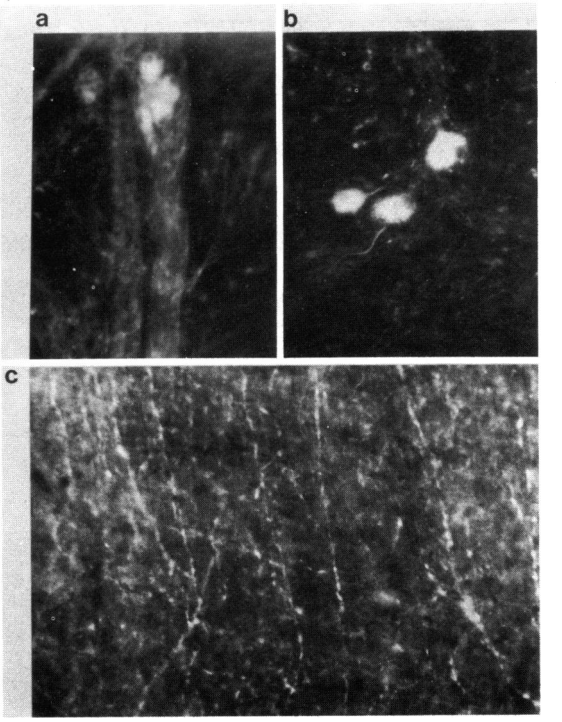

Figure 9 Quinacrine fluorescence in the rabbit portal vein after chemical sympathectomy. (a) Intensely stained cell bodies with unstained nuclei are seen associated with a nerve bundle. Fluorescent micrograph of whole mount × 137. (b) Fluorescent ganglion (containing three nerve cell bodies, diam. 20 to 40 μm). Note a fine fibre projecting from one nerve cell body. Fluorescent micrograph × 117. (c) Fine varicose nerve fibres are seen as in Figure 8a. Fluorescent micrograph × 78.

caused a 5 fold (5.44 ± 0.70; mean ± s.e., $n = 8$) increase above prestimulation levels (Figure 7a). Thus, approximately half of the radioactive adenyl compounds released appeared to be from adrenergic neurones. This is in agreement with the findings of Su (1975).

In the presence of tetrodotoxin (1×10^{-6} to 2×10^{-6} M), there was no significant release of tritium above prestimulation levels. Release is unlikely to be due to muscle movement, since in many preparations the veins were relaxed and thus showed negligible changes in tension during the response to non-adrenergic inhibitory nerve stimulation.

Histochemistry In the rabbit portal vein, quinacrine fluorescence histochemistry revealed a dense green plexus of fine varicose nerve fibres on the outer surface of the longitudinal muscle coat (Figure 8a). Intramural ganglia (4.6 per 1 to 1.5 cm length tissues) containing brightly fluorescent spherical cell bodies (diam. 20 to 40 μm) were also observed (Figures 9a & b). These were occasionally associated with weakly fluorescent nerve fibres running in bundles. Quinacrine-positive neural elements persisted after chemical sympathectomy with 6-hydroxydopamine (Figure 9a, b, c). Quinacrine histochemistry of transverse sections have revealed a second nerve plexus between the circular and longitudinal muscle coats, in addition to the plexus illustrated at the adventitial-medial junction in stretch preparations. A few nerve fibres appeared to traverse the longitudinal muscle coat between the two plexuses. The ganglion cells (diam. 17 to 20 μm) were located in the adventitia. These relationships are illustrated diagrammatically in Figure 10.

With the catecholamine fluorescence technique, a dense green adrenergic nerve plexus but no ganglia, was revealed on the outer surface of the longitudinal

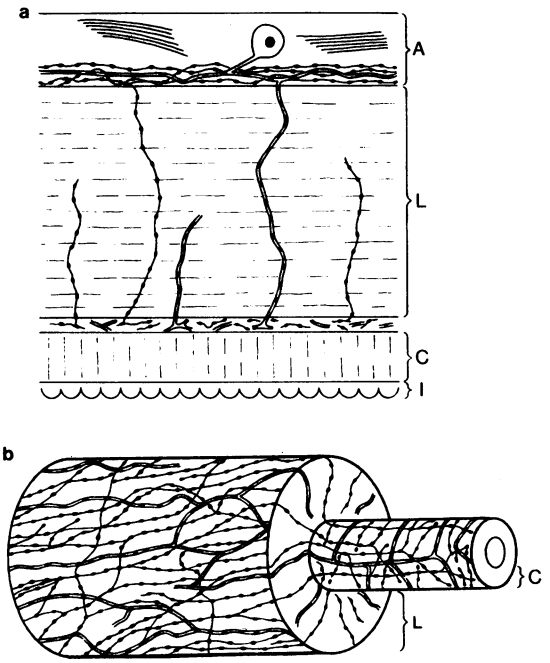

Figure 10 Schematic representations of the innervation of the rabbit portal vein. (a) Longitudinal section showing the adventitia (A); the media consisting of the longitudinal (L) and circular (C) muscle coats, and the intima (I). Both the adrenergic (—●—) and non-adrenergic (■ ■ ■) nerves are found on the adventitial-medial border and in between the two muscle coats. Both types of plexuses are connected by axons which traverse the longitudinal muscle coat. Quinacrine nerve cell bodies are found in the adventitia layer; nerve fibres from these cells join the peri-arterial plexus. Some bundles of preterminal fibres are located in the middle and outer levels of the adventitia. (b) Three-dimensional representation of the relationship of the nerve plexuses to the two muscle (longitudinal and circular) layers. Note the density of the adrenergic (—●—) plexus is greater than the non-adrenergic (■ ■ ■) plexus.

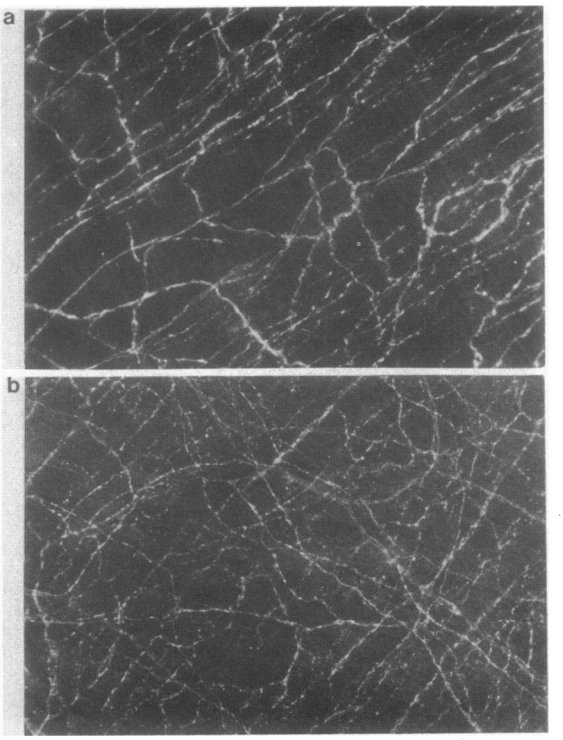

Figure 11 Catecholamine fluorescence in rabbit and guinea-pig portal veins. (a) Rabbit portal vein. A dense adrenergic nerve plexus is demonstrated. Fluorescent micrograph of whole mount × 113. (b) Guinea-pig portal vein. A dense adrenergic nerve plexus is seen with paraformaldehyde treatment. Fluorescent micrograph of whole mount × 106.

coat (Figure 11a). This plexus was absent in 6-hydroxydopamine-treated animals.

Measurements made of adrenergic and quinacrine-positive nerves show that there are approximately 3 to 7 times more adrenergic nerves. Adrenergic nerves appeared either singly or in bundles (diam. 5 to 15 μm) in more than one plane and monoamine fluorescence was confined to the varicosities (about 10 per 100 μm of terminal fibres). The quinacrine-positive nerve plexus consisted predominantly of single nerves confined to one plane; intense fluorescence was found in varicosities (about 10 per 100 μm), but some fluorescence was usually also seen in intervaricose regions. A few nerve bundles (20 to 30 μm in diameter) were seen deep in the adventitia; they showed weak fluorescence and probably contained pre-terminal fibres.

No acetylcholinesterase activity was demonstrated.

Guinea-pig portal vein

Responses to intramural nerve stimulation and noradrenaline The guinea-pig portal vein was spontaneously active, and contracted in response to intramural nerve stimulation in a frequency-dependent manner (1 to 40 Hz). The contractions reached a maximum amplitude at 30 to 40 Hz frequency (Figure 12a) and were mimicked by exogenous noradrenaline (6×10^{-8} to 10^{-5} M).

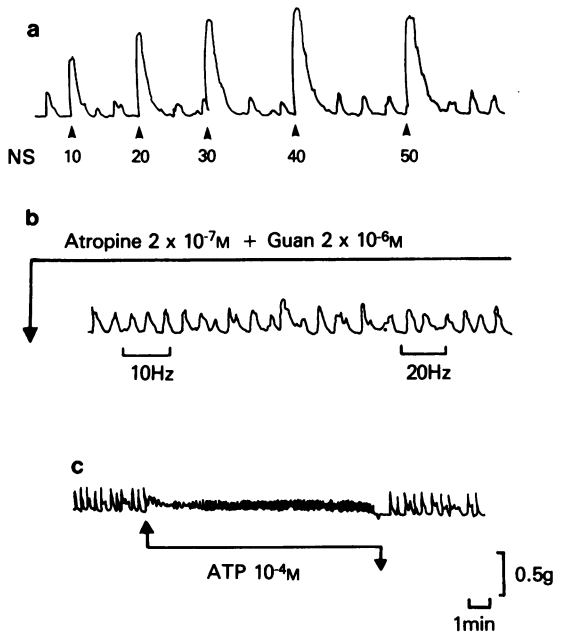

Figure 12 Responses of the guinea-pig portal vein: (a) to intramural nerve stimulation (NS ▲, at different frequencies (Hz), 0.3 ms pulse duration and supramaximal voltage) for 10 s; (b) response to nerve stimulation, at 10 and 220 Hz 30 min after the administration of atropine (2×10^{-7} M) and guanethidine (2×10^{-6} M) (c) effect of continuous infusion of ATP (10^{-4} M).

Effects of drugs on the responses to intramural nerve stimulation and noradrenaline

α-Adrenoceptor antagonists Low concentrations of phentolamine (5×10^{-6} to 10^{-5} M) and phenoxybenzamine (10^{-5} M), which did not change the basal tone of the preparation, each abolished the responses of the portal vein to intramural nerve stimulation and to exogenous noradrenaline (6×10^{-8} to 10^{-5} M). No inhibitory responses were unmasked, even when the tone had been raised with ergotamine.

Guanethidine Contractions of the guinea-pig portal vein in response to intramural nerve stimulation were abolished by the adrenergic neurone blocker, guanethidine (10^{-6} M). After treatment with guanethidine, there were no residual excitatory or inhibitory responses to intramural nerve stimulation (Figure 12b), even when the basal tone of the tissue was raised by low concentrations of histamine or noradrenaline.

Tetrodotoxin Tetrodotoxin (10^{-6} M) abolished the excitatory responses of the guinea-pig portal vein to intramural nerve stimulation, without changing the basal tone of the preparation.

Responses to ATP ATP (10^{-6} to 10^{-3} M) caused an inhibition of spontaneous activity as well as a small increase in basal tone (Figure 12c). When the tone of the vein was raised with a low concentration of histamine (9×10^{-8} M), ATP elicited a small contraction followed by a relaxation. In preparations of low basal tone, ATP, at high concentrations (10^{-5} to 10^{-3} M), elicited a contraction.

Release of ^{3}H-adenyl compounds After incubation of the guinea-pig portal veins in [^{3}H]-adenosine, intramural nerve stimulation (at 30 Hz, 0.3 ms, and supramaximal voltage for 60 s) in the absence of any drugs, caused a 6 fold (6.04 ± 1.64; mean ± s.e., $n = 6$) increase in ^{3}H-adenyl compounds above prestimulation levels (Figure 7b). In veins that were treated with guanethidine (6×10^{-6} M), intramural nerve stimulation at similar parameters produced no increase in ^{3}H-adenyl compounds in the superfusion fluid.

Histochemical studies In the guinea-pig portal vein, no quinacrine-positive neural elements (either fibres or cell bodies) were observed in whole mounts (Figure 8b) or in tissue sections after sympathectomy. However, with paraformaldehyde treatment, a dense catecholamine containing nerve plexus, but no nerve cell bodies, was demonstrated on the adventitial-medial border (Figure 11).

The guinea-pig portal vein did not exhibit acetylcholinesterase activity.

Discussion

In agreement with the findings of other workers (Hughes & Vane, 1967; 1970; Su, 1975) an inhibitory response resistant to α- and β-adrenoceptor blockade, chemical sympathectomy with 6-hydroxydopamine, and cholinoceptor blockade, was observed in the rabbit portal vein. This non-adrenergic, non-cholinergic relaxation of the longitudinal muscle coat is mediated by nerves as it is abolished by tetrodotoxin. In the guinea-pig portal vein, there have been no reports of a non-adrenergic, non-cholinergic innervation. We found that after the administration of the adrenergic neurone blocker, guanethidine, and of atropine, there was no residual excitatory or inhibitory response. The portal veins of the rabbit and guinea-pig thus provided a valuable comparative system for study of the non-adrenergic, non-cholinergic transmitters.

In view of the evidence that non-adrenergic, non-cholinergic nerves supplying the gastrointestinal tract and bladder may be purinergic (see Burnstock *et al.*,

1970; 1978a; Burnstock, 1972; 1975b) and of the demonstration by Hughes & Vane (1967) that the inhibitory responses of the rabbit portal vein were mimicked by ATP, it was of interest to compare the release of ^{3}H-adenyl compounds by intramural nerve stimulation from the rabbit and guinea-pig portal veins after preloading with [^{3}H]-adenosine. Su (1975) has shown that [^{3}H]-adenosine is taken up by the rabbit portal vein, transformed into ^{3}H-nucleotides, mainly [^{3}H]-ATP, and stored in this form. In our experiments, tritium released from the rabbit portal vein during intramural nerve stimulation was from both adrenergic and non-adrenergic, non-cholinergic nerves, since the release was reduced by nearly 50% after adrenergic neurone blockade with guanethidine, and the remainder was abolished by tetrodotoxin. Similar results have been reported by Su (1975).

Release of ATP together with noradrenaline from adrenergic nerves has been claimed previously (Su, Bevan & Burnstock, 1971; Su, 1975; Langer & Pinto, 1976; Westfall, Stitzel & Rowe, 1978). Based on fluorescent histochemical localization of monoamines and quinacrine, it was calculated that there are about 3 to 7 times more adrenergic than non-adrenergic varicosities in the rabbit portal vein. During intramural nerve stimulation, ATP is released in equal amounts from the adrenergic and non-adrenergic nerves. Assuming that the transmitter is released from the same proportion of varicosities of both fibre types during intramural nerve stimulation, this means that any one adrenergic varicosity releases 3 to 7 times less ATP than a purinergic varicosity. If it is assumed that the amount of ATP released on purinergic nerve stimulation is the same as the amount of noradrenaline released on adrenergic nerve stimulation, the present finding is consistent with the proportion of catecholamine to ATP stored in adrenergic nerves (4 to 10:1; see Burnstock & Costa, 1975).

It has always been difficult to ascertain whether the source of nucleotide release is from nerves or secondarily from muscle as a result of changes in tension. In these experiments, the portal vein was fully relaxed after incubation in [^{3}H]-adenosine, so that there were negligible changes in tension during the response to stimulation of the non-adrenergic, non-cholinergic nerves. Further, it has previously been shown that the release of nucleotides from guinea-pig taenia coli and bladder during non-adrenergic, non-cholinergic nerve stimulation is from nerves and not muscle (Burnstock, Cocks, Kasakov & Wong, 1978b; Rutherford & Burnstock, 1978).

In contrast, in the guinea-pig portal vein the release of tritium during nerve stimulation was abolished by guanethidine. Thus, the only source of ^{3}H-adenyl compounds release in the guinea-pig portal vein is adrenergic nerves. This release was also blocked by tetrodotoxin.

Fluorescent histochemical localization of quinacrine revealed a distinct population of nerve fibres and ganglia in the rabbit portal vein, while no such nervous elements were found in the guinea-pig. In contrast to adrenergic nerves, they were resistant to chemical sympathectomy with 6-hydroxydopamine. Thus, the quinacrine-positive nerves are not adrenergic. They are not likely to be cholinergic since acetylcholinesterase activity was not exhibited. Furthermore, quinacrine-positive nerves are absent from tissues such as the iris, which does not contain a non-adrenergic, non-cholinergic nerve component, but which contains both adrenergic and cholinergic nerves (Burnstock *et al.*, 1978a). There is evidence that quinacrine binds to ATP (Irvin & Irvin, 1954) and also to regions of DNA rich in adenine and thymine (Ellison & Barr, 1972). More recent evidence shows an accumulation of quinacrine in granules containing high levels of ATP in blood platelets (Lorez, Da Prada & Launay, 1977), and the adrenal medulla (Olson *et al.*, 1976). Furthermore the distribution of quinacrine-positive fibres in the gut and bladder is comparable to that of purinergic nerves based on pharmacological evidence (Burnstock, 1975a; Olson *et al.*, 1976; Burnstock *et al.*, 1978a).

The presence of quinacrine-positive nerve cell bodies within the wall of the rabbit portal vein is of considerable interest, since there are few reports of intramural ganglion cells in blood vessels (see Honig & Frierson, 1976). This finding is supported by the observation of Hughes & Vane (1970) that the ganglion stimulant, nicotine, produces the non-adrenergic relaxation and that hexamethonium reduces the response to transmural stimulation. The origin of the preganglionic fibres is not known, but by analogy with the preganglionic fibres to intramural purinergic nerves in the stomach, bladder, lung and gall bladder, they seem likely to be parasympathetic.

The relaxations of the rabbit portal vein in response to ATP were not usually as large as those produced by intramural nerve stimulation; further, in some portal veins ATP produced a rapid relaxation followed by a slower relaxation, while in others, it produced only a slow relaxation. These responses to ATP may be explicable in terms of a differential location of purinergic receptors. Quinacrine fluorescence histochemistry showed plexuses present both at the adventitial-medial junction and between the circular and longitudinal muscle layers. Receptors on the outer surface of the muscle coat may be more easily accessible to exogenous ATP and thus mediate a rapid relaxation. Other ATP receptors may be present between the muscle layers. To gain access to these receptors, exogenous ATP would have to diffuse through muscle layers and would be subject to rapid breakdown. This may explain both the necessity to

use high concentrations of ATP and the secondary slow relaxation sometimes observed.

The prostaglandin synthesis inhibitor, indomethacin, potentiated the relaxations of the rabbit portal vein to non-adrenergic, non-cholinergic nerve stimulation and to ATP. Prostaglandins E_1 and E_2 contract the rabbit portal vein, and Hughes & Vane (1967) showed that prostaglandin $F_{2\alpha}$ also causes contractions. Since ATP has been shown to induce prostaglandin synthesis (Needleman, Minkes & Douglas, 1974), this potentiation of the non-adrenergic response in the rabbit portal vein may be due to inhibition of prostaglandin synthesis by indomethacin.

While the good correlation between the histochemical radioactivity and indomethacin studies suggests that ATP may be the non-adrenergic, non-cholinergic neurotransmitter in the rabbit portal vein, experiments with some of the other drugs known to modify purinergic transmission in the gut did not prove useful. For example, the 2-substituted imidazolines, phentolamine and antazoline, in high concentrations (3.5×10^{-5} to 1.8×10^{-4} M), which have been shown to antagonize purinergic nerve responses and ATP in the guinea-pig taenia coli (Satchell *et al.*, 1973) caused a loss of basal tone which masked their effects on the responses to both ATP and non-adrenergic nerve stimulation in the rabbit portal vein. Similarly dipyridamole, an adenosine uptake inhibitor, in concentrations which have been reported to potentiate the responses to both ATP and purinergic nerve stimulation in the guinea-pig taenia coli (Satchell *et al.*, 1972), was ineffective on both responses of the rabbit portal vein. This is probably because adenosine is not as potent as ATP in relaxing the portal vein, and also because the drug caused a loss of tone, which may have masked its effects.

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