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SUBSTANCE P MEDIATES NEUROGENIC VASODILATATION IN EXTRINSICALLY DENERVATED GUINEA-PIG SUBMUCOSAL ARTERIOLES

BY J. J. GALLIGAN*, MING-MING JIANG, K.-Z. SHEN AND ANNMARIE SURPRENANT

From the Vollum Institute, Oregon Health Sciences University, Portland, OR 97201, USA

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SUMMARY

1. Arteriolar diameter was measured using an optical method in preparations of guinea-pig submucosal plexus *in vitro*. Electrical stimulation of one of more neurones in ganglia of the submucosal plexus causes a cholinergic vasodilatation in normal animals. The vasomotor innervation to the arterioles was studied in guinea-pigs in which the extrinsic nerves to the intestine had been removed. Tissues were processed for immunohistochemistry after the *in vitro* experiments.

2. Extrinsic denervation resulted in complete loss of catecholamine fluorescence, NPY (neuropeptide Y) and CGRP (calcitonin gene-related peptide) immunofluorescence around the blood vessels and no neurogenic vasoconstriction was observed up to 60 days post-denervation. Vasodilatation in response to ganglionic stimulation was increased; smaller arterioles (outside diameter $< 40\ \mu m$) showed a greater enhancement of neurogenic vasodilatation than larger arterioles.

3. Nerve-evoked vasodilatations were only partially inhibited by muscarinic antagonists at 30–60 days after extrinsic denervations.

4. The non-cholinergic neurogenic vasodilatation was abolished by the substance P antagonists, spantide, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]substance P and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]substance P. These antagonists did not alter the cholinergic vasodilatation in normal or extrinsically denervated arterioles.

5. Exogenous substance P dilated all submucosal arterioles; the concentration which produced half-maximal vasodilatations was 2·5 mM in both normal and extrinsically denervated arterioles. Substance P antagonists inhibited the vasodilatation caused by substance P at concentrations similar to those needed to block nerve-mediated vasodilatation.

6. There was a strong correlation between the finding of non-cholinergic vasodilatation in response to ganglionic stimulation, and the presence of substance P-immunoreactive fibres running from ganglion to arteriole. This correlation did not exist for VIP (vasoactive intestinal peptide).

* Permanent address: Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI, USA.

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7. These results suggest that intrinsic intestinal substance P-containing nerve fibres supply submucosal arterioles after sympathetic efferents and sensory afferents are removed. Stimulation of these nerves releases substance P to produce arteriolar dilatation.

INTRODUCTION

Several vasoactive substances can be detected in nerve fibres associated with blood vessels, including noradrenaline, substance P (SP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) (see Burnstock & Griffith, 1988). Which of these substances (except noradrenaline) provide a functional innervation to a given vascular network has been a particularly difficult question to study for resistance vessels, because measurement of vessel diameter or luminal pressure in isolated preparations of small arterioles ($< 100\ \mu$m outside diameter) has been technically difficult or impossible. The recent development of a simple and convenient method for on-line tracking of vessel diameter (Neild, 1989) has provided the opportunity to examine vasomotor innervation of previously inaccessible vascular networks.

In the preceding paper we demonstrated that functional vasodilator innervation to submucosal arterioles in the guinea-pig small intestine is provided by nerve fibres of cholinergic neurones residing within the submucosal plexus (Neild, Shen & Surprenant, 1990). No evidence for a functional non-adrenergic, non-cholinergic innervation to these vessels was obtained even though immunohistochemical studies have demonstrated that nerve fibres containing SP, CGRP and VIP are associated with these vessels (Furness & Costa, 1987). However, alterations in the neuropeptide content of nerves supplying these vessels take place after surgical removal of their extrinsic sympathetic and sensory nerve supply (Galligan, Costa & Furness, 1988). In normal guinea-pig intestine, in addition to sympathetic nerve fibres, there are nerve fibres arising from cell bodies in dorsal root ganglia which contain both SP and CGRP. The SP/CGRP-containing fibres are associated with every submucosal arteriole in normal intestine. At 1–2 months after denervation nerve fibres which contain SP but not CGRP supply most arterioles (Galligan *et al.* 1988). The purpose of the present study was to determine whether extrinsic denervation might also lead to alterations in the functional innervation of submucosal arterioles. We used video-monitoring of vessel diameter, electrical stimulation of the nerve fibres present in the submucosa and immunohistochemical methods to examine this question.

METHODS

Methods of tissue preparation, focal extracellular stimulation of ganglia, drug application via superfusion or pressure ejection, video-monitoring of vessel diameter were as described in the preceding article (Neild *et al.* 1990).

Extrinsic denervation. Guinea-pigs were anaesthetized (pentobarbitone, 15 mg/kg and Innovar®, 0·5 ml/kg administered intramuscularly) and a loop of small intestine was exposed via a mid-line abdominal incision. The nerve supplying that loop of intestine were crushed using watchmakers forceps (see Furness & Costa, 1978) and the animals were allowed to recover for periods of between 30 to 60 days. The animals were then killed by decapitation and the denervated segments of intestine were cut into four to six pieces for histochemical and physiological analyses. Unoperated

segments of intestine from the same animal were obtained for control studies. Extrinsic denervation of the intestinal loops was verified in one piece of submucosa by using the glyoxylic acid method for localizing neuronal stores of catecholamines (Furness & Costa, 1975). The remaining pieces of submucosa were used for functional studies and subsequent immunohistochemical localization of neuropeptides.

Immunohistochemistry. All preparations used in functional studies were processed for immunohistochemical localization of neuropeptides. These tissues were fixed overnight at 4 °C in 2% formaldehyde/15% picric acid in 0·1 M-phosphate buffer (pH 7·0). The tissues were subsequently washed 3–4 times for 10 min in dimethyl sulphoxide and were stored in phosphate-buffered saline (PBS, 0·01 M, pH 7·0). Localization of neuropeptides was accomplished by incubating preparations, in a humid chamber, overnight with diluted antisera raised against SP, VIP, CGRP or NPY. The antisera, host species, the dilutions used and the antisera supplier were as follows: VIP (No. 7913), rabbit, 1:200 was a gift from Dr John Walsh, Los Angeles, CA; SP (NC1/34HL), rat monoclonal, 1:200, purchased from Bioproducts for Science, Indianapolis, IN; SP (No. 962) rabbit, 1:400, purchased from Chemicon International, Los Angeles, CA; CGRP (No. 1842) rabbit, 1:200, purchased from Amersham, Arlington Heights, IL; NPY (No. 1702), rabbit, 1:200, purchased from Amersham. In most experiments, the rat anti-SP antiserum was used in combination with a rabbit antiserum raised against one of the other antigens for simultaneous localization of SP with each of the other neuropeptides. The primary antisera were localized using fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobin G (IgG) and goat anti-rabbit tetramethylrhodamine isothiocyanate (TRITC)-conjugated IgG raised against rat and rabbit IgG respectively (both used at 1:80 final dilution and purchased from Chemicon International, Los Angeles, CA). Preliminary studies were done to determine the optimal dilution for each antiserum and to ensure that immunostaining was abolished by pre-incubating the primary antiserum with the appropriate authentic peptide (1 μM-peptide concentration). In addition, all primary and secondary antisera were tested in various combinations to ensure that there were no inappropriate cross-reactivities between primary antisera, secondary antisera or between primary and secondary antisera. Preparations were mounted in buffered glycerol (pH 8·6) for fluorescence microscopy.

Analysis of vasomotor responses. Vasodilatations produced by nerve stimulation or pressure-ejection of drug were expressed as the 90% area under the curve of the dilatory response, as described in the preceding paper. Vasoconstrictions are expressed as percentage of maximum response produced by high (60 mM) potassium solution or by maximum concentration of U46619. SP and SP antagonists were applied by superfusion; all antagonists were present in the bathing fluid for 7 min prior to application of agonist. All concentration response curves were obtained by cumulative additions of the agonist (e.g. as in Fig. 7). Data are expressed as means $\pm$ S.E. of mean.

The extent of projections of SP- and VIP-containing nerve fibres from the ganglion to the monitored arteriolar segments was semi-quantitatively evaluated using a scale which ranged from 0 (no peptide-containing fibres emerging from the ganglion to the arteriole; e.g. Fig. 5) to 3 (many fibres projecting from the ganglion and forming a varicose perivascular plexus around the monitored segment of blood vessel; e.g. Fig. 4). The scale is described in detail in the legend to Fig. 6. The evaluation was performed in a blind fashion such that the scorer did not know the results of the physiological experiment performed on any particular ganglion-to-arteriole set.

The peptide-containing nerve fibres supplying blood vessels and in ganglia-blood vessel projections frequently wrapped around the circumference of the blood vessel so that a single nerve fibre would enter and leave the plane of focus. Thus, one photomicrograph would not yield an accurate and complete representation of the extent of SP- or VIP-containing nerves associated with an arteriolar segment. To circumvent this problem, several photomicrographs were taken at different focal planes and the nerve fibres associated with the ganglion and the arteriole were redrawn using a transparency (e.g. Figs 4 and 5).

Effectiveness of denervation. All results on denervated arterioles were obtained from preparations in which extrinsic denervation had been performed 30–60 days previously. None of these preparations (nineteen preparations from eleven animals) contained any sympathetic nerve fibres as evidenced by absence of both glyoxylic acid-induced catecholamine fluorescence in the entire submucosal plexus (Furness & Costa, 1975), and NPY immunofluorescent fibres associated with blood vessels were absent from whole-mounts of arterioles (Costa & Furness, 1984). We also examined whether sensory fibres were present, as a result of incomplete denervation and/or reinnervation from central sources, by carrying out double-labelling procedures with SP and CGRP antibodies. In normal submucosal preparations, SP and CGRP were co-localized in dense networks

of nerve fibres surrounding every arteriole, as demonstrated previously (Gibbins, Furness, Costa, MacIntyre, Hillyard & Girgis, 1985; Galligan *et al.* 1988). No CGRP-containing nerve fibres associated with submucosal arterioles were observed in the extrinsically denervated preparations ($n = 11$) even though extensive networks of SP-containing fibres were present in all denervated arterioles (see Results). Therefore it was concluded that the extrinsic denervations were successful and that there was no significant reinnervation by either sympathetic fibres or sensory fibres during the time course of our experiments.

Drugs. The following drugs were used: substance P (SP), [D-Arg^1, D-Trp^{7,9}, Leu^{11}]substance P (spantide), [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]substance P ([APTTL]SP), [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]substance P ([APhTTL]SP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP, Sigma and Peninsula); (−)scopolamine HCl, 1-phenylephrine, DL-muscarine chloride, eserine and tetrodotoxin (Sigma); guanethidine (CIBA), 9,11-dideoxy-11α, 9α-epoxy-methano-prostaglandin F2$_\alpha$ (U46619, Upjohn); 4-diphenylacetoxy-*N*-methyl-piperidine methiodine (4-DAMP, Research Biochemicals Inc.); (11[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-)-one (AFDX-116) and pirenzepine provided by Boehringer.

RESULTS

Vasoconstriction following extrinsic denervation

Absence of neurogenic vasoconstriction

Electrical stimulation (2–20 Hz for 10 s) of a submucosal ganglion never elicited a vasoconstriction in any arteriole in which extrinsic denervation had been performed ($n = 36$), although identical ganglionic stimulation in normal preparations produced a vasoconstriction in approximately 40% of the trials ($n = 12$; see also Neild *et al.* 1989). When the focal stimulating electrode was placed on the arteriole itself, stimulation also did not cause any vasoconstriction up-stream or down-stream of the site of stimulation; this type of stimulation always constricted the vessel in normal preparations.

Vasoconstriction to exogenously applied substances

We examined whether extrinsic denervation might alter the sensitivity to exogenously applied vasoconstrictor substances by determining concentration–response curves for noradrenaline in normal and extrinsically denervated preparations. The results from these experiments are shown in Fig. 1 from which it can be seen that extrinsic denervation did not alter the concentration range over which noradrenaline produced arteriolar vasoconstriction; EC_{50} values for noradrenaline-induced constriction were 600 nM in normal and in denervated arterioles. Similar results were obtained with the prostaglandin analogue, U46619; 70 nM-U46619 produced 35–60% of maximum vasoconstriction in both normal and extrinsically denervated preparations ($n = 3$). Noradrenaline, phenylephrine, U46619 and high external potassium solution (60 mM) produced the same maximum vasoconstriction which, in all arterioles examined (outside diameters ranging from 25–90 μm), was complete occlusion of the lumen.

Vasodilatation following extrinsic denervation

Neurogenic vasodilatations

Submucosal arterioles were pre-constricted with U46619, using a concentration (70–285 nM) which produced approximately 80% of the maximum vasoconstriction.

Focal stimulation of a submucosal ganglion (10 Hz for 10 s) produced arteriolar dilatation in twenty-two out of thirty-three (67%) trials in normal preparations (Fig. 2*A*); this percentage is approximately the same as that observed in our previous experiments (62%; Neild *et al.* 1989). Similar ganglionic stimulation in

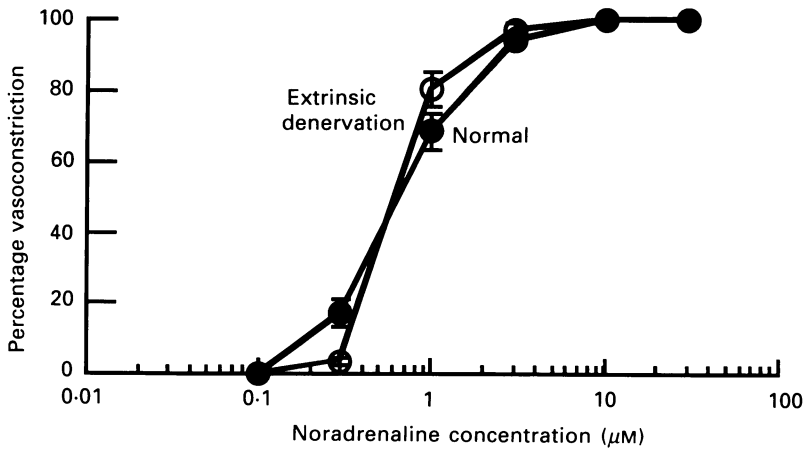

Fig. 1. Extrinsic denervation does not alter the sensitivity of submucosal arterioles to noradrenaline. Vasoconstriction (as percentage of response to 60 mM-potassium) plotted as a function of noradrenaline concentration in normal preparations (●) and preparations from extrinsically denervated preparations (○). Concentration of noradrenaline which produced half the maximum response (EC_{50}) was 600 nM in both cases. The maximum response was occlusion of the lumen. Each point is the mean ± S.E.M.; $n = 4$ for all points.

extrinsically denervated preparations produced a vasodilatation in a significantly larger proportion of trials (29/30, 97%; $\chi^2 = 8{\cdot}97$, $P < 0{\cdot}005$). The vasodilatation itself was significantly increased (Figs 2*B* and 3*B*) and it became apparent that these effects of extrinsic denervation were largely confined to the smaller arterioles (Fig. 3*A*). Therefore, we compared the effects of ganglionic stimulation on vessels less than, and greater than, 40 μm outside diameter. We chose this division because it represents approximately the mid-point of the range of diameters of submucosal arterioles in the guinea-pig small intestine *in vitro* (e.g. outside diameters ranged from 15–100 μm). There was no significant difference in the vasodilatation, measured as percentage of pre-constricted diameter, produced by nerve stimulation in vessels in either size group in normal preparations (Fig. 3*B*). In extrinsically denervated preparations, the neurogenic vasodilatations for arterioles of both sizes was greater than that observed in control tissues. The vasodilatation in arterioles less than 40 μm diameter was 160% greater than in normal preparations while the larger arterioles responded with an average vasodilatation 75% greater than similar-sized, non-denervated arterioles (analysis of variance, $F = 19{\cdot}2$; d.f. = 1,40; $P < 0{\cdot}001$; Fig. 3*B*). The effect of denervation on the vasodilatory response was significantly greater on the smaller arterioles when compared to the larger vessels ($F = 8{\cdot}4$; d.f. = 1,40; $P < 0{\cdot}01$).

Previously, we found that nerve-evoked vasodilatation of submucosal arterioles was abolished by muscarinic antagonists (Neild *et al.* 1990); similarly, in these experiments ganglionic stimulation produced vasodilatations in control arterioles

which were completely blocked in the presence of pirenzepine (1 μM, $n = 9$) or 4-DAMP (200 nM, $n = 10$) (Figs 2*A* and 3*C*). After extrinsic denervation, complete block of the vasodilatation was observed in only 24% (4/17; $\chi^2 = 26{\cdot}8$, 3 d.f., $P < 0{\cdot}001$) of the arterioles even with much higher concentrations of muscarinic

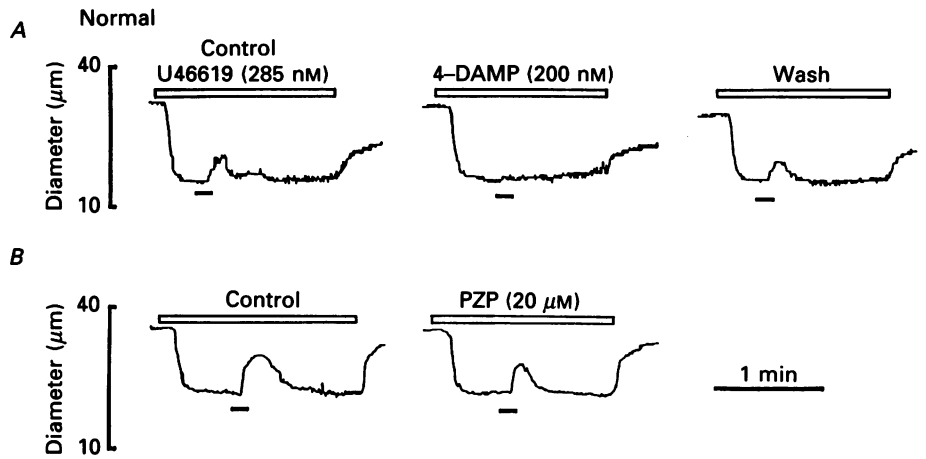

Fig. 2. Examples of a typical neurogenic cholinergic vasodilatation in a normal submucosal arteriole (*A*) and the non-cholinergic vasodilatation observed in extrinsically denervated arterioles (*B*). The prostaglandin analogue, U46619 (present in the superfusing solution for duration indicated by bar above each trace) was used to pre-constrict the vessel and a submucosal ganglion was stimulated (10 Hz for 10 s) at the time indicated by the filled bar below each trace. In the experiment illustrated in *A* the muscarinic antagonist, 4-DAMP, abolished the nerve-evoked vasodilatation (middle record) in a reversible manner (right-hand record); complete blockade of nerve-evoked vasodilatation was observed in all normal arterioles in the presence of 200 nM 4-DAMP or 2 μM-pirenzepine. *B*, a similar experiment performed on a preparation in which the extrinsic sympathetic and sensory fibres had been surgically interrupted 40 days previously reveals a nerve-evoked vasodilatation which was only partially depressed in the presence of 20 μM-pirenzepine. Note that the vasodilatation (as indicated by the area under the vasodilatory response) was substantially larger in the extrinsically denervated arteriole. Outside diameters of arterioles were approximately equivalent (resting diameter 33 μm in arteriole from *A* and 37 μm in arteriole from *B*.

antagonists (e.g. 20 μM-PZP, 2 μM-4-DAMP, 2 μM-scopolamine). The effect of extrinsic denervation on the presence of cholinergic *versus* non-cholinergic vasodilatation was not significantly different between the smaller and larger arterioles, with nine of ten small vessels showing non-cholinergic vasodilatation and three of seven large arterioles displaying a non-cholinergic neurogenic vasodilatation ($\chi^2 = 5{\cdot}4$, 3 d.f.; $P > 0{\cdot}05$; Fig. 3*C*).

Blockade by substance P receptor antagonists

Figure 4 shows the results from one experiment in which the non-cholinergic vasodilatation was abolished by the substance P antagonist spantide. In this experiment, ganglionic stimulation evoked a vasodilatation which was inhibited by 47% by 4-DAMP (1 μM); addition of spantide (6 μM) reversibly abolished the 4-DAMP-resistant vasodilatation (Fig. 4). Lower concentrations (0·5 and 1 μM) of spantide also completely inhibited the non-cholinergic vasodilatation ($n = 3$). Two

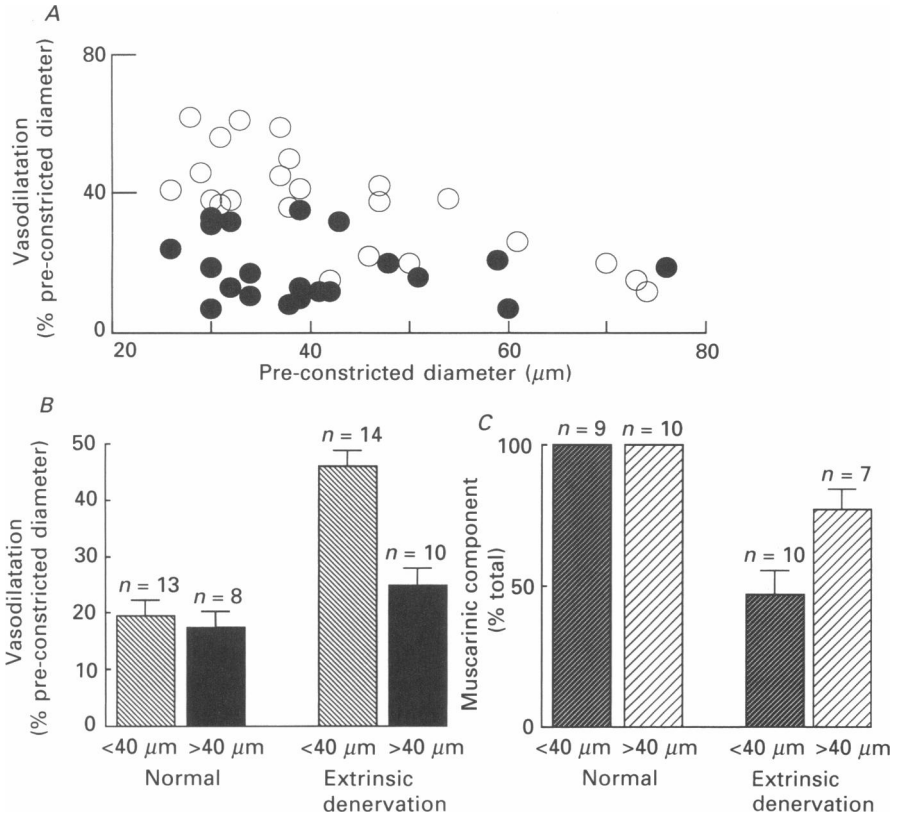

Fig. 3. Changes in nerve-evoked vasodilatations which occurred in submucosal arterioles consequent to removal of the extrinsic sympathetic and sensory nerve supply. *A*, scatter diagram of the vasodilatation (measured as the 90% area under the curve of the dilatory response and expressed as percentage of pre-constricted diameter) in normal arterioles (●) and in extrinsically denervated preparations (○). *B*, summary of neurogenic vasodilatations in normal and denervated arterioles; the vasodilatation was the same in both small ($< 40\ \mu m$) and larger ($> 40\ \mu m$) vessels while the vasodilatations produced in extrinsically denervated arterioles were significantly increased ($P < 0{\cdot}005$) over normal preparations; this increased responsiveness was most prominent in the smaller vessels. All vasodilatations were evoked by stimulating an individual submucosal ganglion with a focal external electrode (10 Hz for 10 s). *C*, inhibition of nerve-evoked vasodilatation by muscarinic receptor antagonists in normal and extrinsically denervated submucosal arterioles; the ordinate is percentage inhibition produced by 200 nM-4-DAMP or 2 μM-pirenzepine in normal preparations and that produced by 2 μM-4-DAMP or 20 μM-pirenzepine in denervated preparations. Muscarinic antagonists produced complete block of vasodilatations in normal preparations while in small arterioles from denervated preparations the cholinergic component was only $45 \pm 8{\cdot}4\%$ and in larger arterioles this muscarinic component was $77 \pm 7{\cdot}2\%$.

other SP receptor antagonists abolished the non-cholinergic vasodilatations: 1 μM-[APhTTL]SP and 1 μM-[APTTL]SP ($n = 3$). No nerve-evoked vasodilatations were observed when both muscarinic and SP receptor antagonists were present; however, we have not examined the effects of stimulation frequencies and durations greater than 20 Hz and 10 s. Neither [APhTTL]SP (1–6 μM) nor [APTTL]SP (1–6 μM) had any direct effect on the diameter of submucosal arterioles ($n = 17$).

Immunohistochemical correlations

We relocated and examined a total of thirteen ganglion–arteriole areas, which were double-labelled for SP and VIP (Figs 4 and 5). The results for the extent of SP- and VIP-containing fibres projecting from ganglia to blood vessels are summarized in

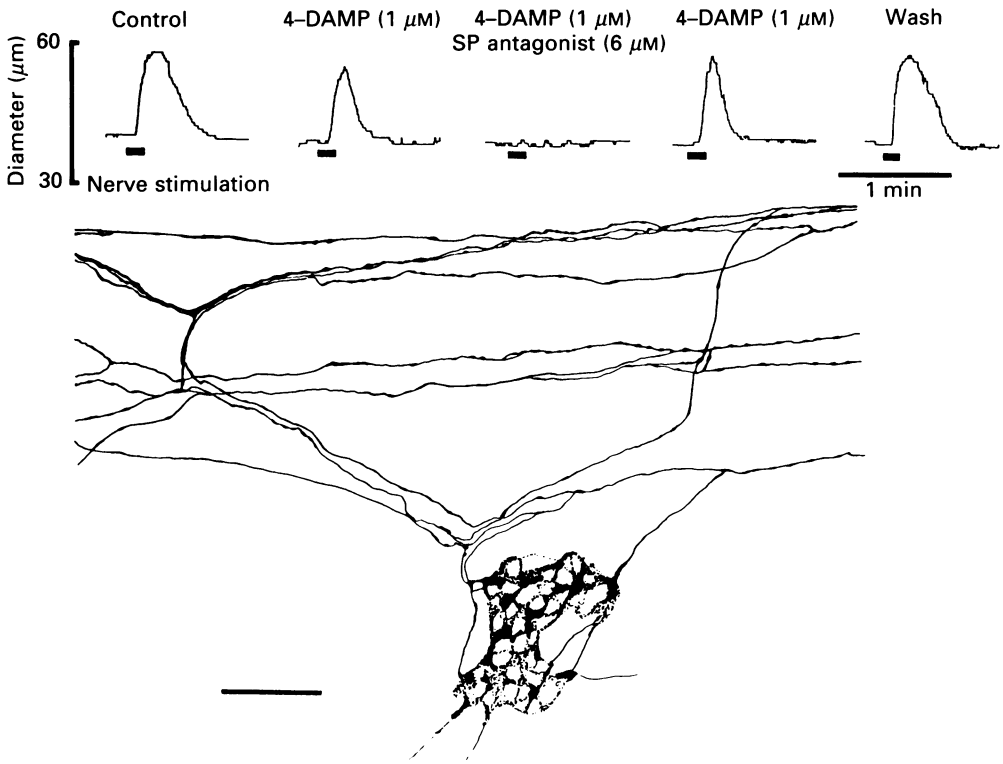

Fig. 4. Blockade of non-cholinergic neurogenic dilatation by the SP receptor antagonist spantide. Recordings were obtained from one arteriolar segment in an extrinsically denervated preparation; nerve stimulation (indicated by bar below each record) produced a vasodilatation which was inhibited by about 50% in the presence of 2 μM-4-DAMP. In the presence of 6 μM-spantide and 4-DAMP no dilatation was evoked by nerve stimulation; the non-cholinergic component returned when spantide was washed out and the additional cholinergic component returned when 4-DAMP was also removed. Arteriole was pre-constricted with 140 nM-U46619; the pre-constricted diameter was 71 μm. After this experiment the preparation was processed for immunohistochemical localization of SP and VIP; the drawing shows the SP immunofluorescence in the ganglion that was stimulated and the arteriolar segment from which the above records were obtained. Calibration bar is 55 μm.

Fig. 6. There was a clear increase in the extent of SP-containing projections to those arterioles showing a non-cholinergic dilatation when compared to those arterioles showing a cholinergic dilatation only. There was no obvious difference between the values obtained for VIP projections and the occurrence of cholinergic or non-cholinergic vasodilatations. No fibres were detected in which SP co-localized with VIP.

Vasodilatations to exogenously applied substances

Muscarine

Muscarine (0·03–3 μM) dilated all submucosal arterioles in both normal ($n = 5$; see Nield *et al.* 1990) and extrinsically denervated preparations ($n = 4$). There was no

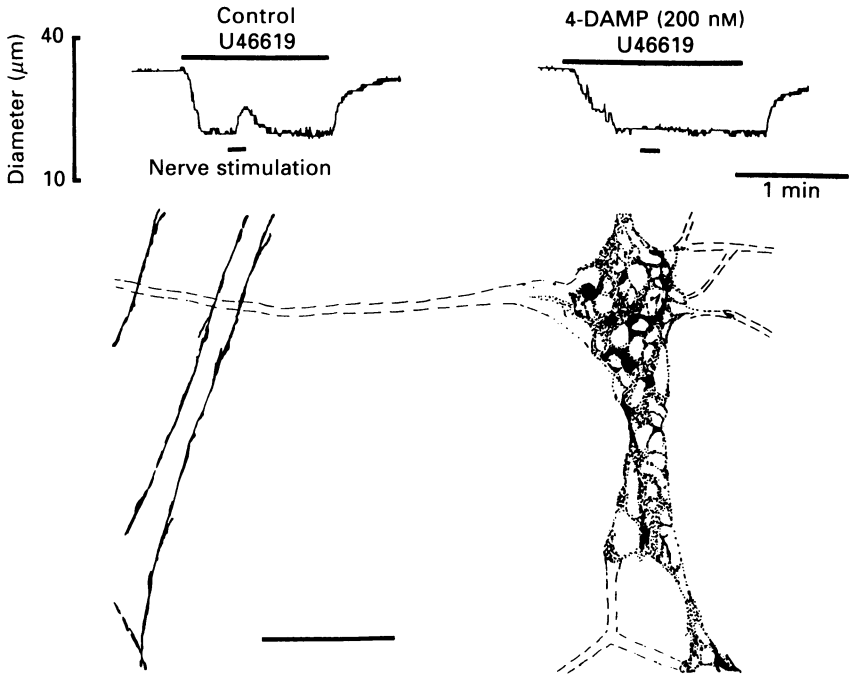

Fig. 5. Example of nerve-evoked vasodilatation recorded from arteriole in an extrinsically denervated preparation which was completely blocked by 200 nM-4-DAMP and the absence of SP-containing nerve fibres projecting from the stimulated ganglion to the monitored arteriolar segment. Vessel was pre-constricted with 142 nM-U46619. Drawing is of SP immunofluorescence of the ganglion/arteriole pair from which the diameter recordings were obtained. Dashed lines indicate fibre tract bundles which could be visualized under bright-field optics. Calibration bar is 50 μm.

significant difference in the concentration–dilatation curves produced by muscarine between normal and denervated arterioles ($n = 4$); there was also no difference in the maximum response produced by muscarine. The SP receptor antagonists, [APTTL]SP (1 and 6 μM) and [APhTTL]SP (6 μM), did not alter the muscarine-induced vasodilatation in normal ($n = 5$) or denervated preparations ($n = 4$).

Substance P

SP also dilated all submucosal arterioles (sixteen arterioles from normal, and eighteen from extrinsically denervated, animals; Figs 7 and 8). The vasodilatation to SP (0·3–30 nM) was sustained during periods of superfusion lasting 2–4 min (Fig. 7). The maximum response to SP (65–100% of pre-constricted diameter) was equivalent to that of muscarine. Figure 8*A* summarizes the SP-induced dilatations in both normal and extrinsically denervated submucosal arterioles; there were no differences and the EC_{50} value was 2·5 nM in either case. The SP-induced vasodilatations were

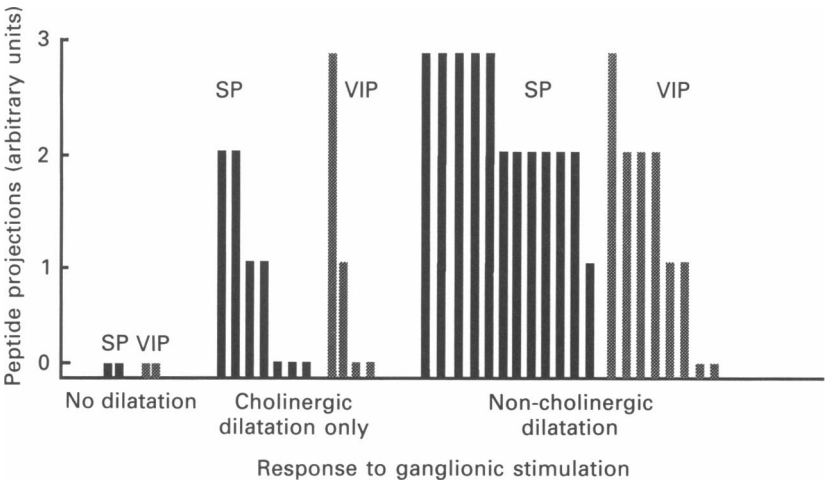

Fig. 6. Correlation between immunohistochemical localization of SP and VIP in nerve fibres projecting from one ganglion to the segment of arteriole which was being monitored during stimulation of the ganglion and response observed in the arteriole. All preparations had been extrinsically denervated 30–60 days prior to carrying out the experiment. A scale of 0–3 was used to evaluate the peptide projections as follows: 0 = no detectable fibres; 1 = one or two faint, fine fibres present in projection; 2 = several bright varicose fibres with some branching around arteriole; 3 = many bright varicose fibres with extensive branching, fibres join a perivascular plexus. The drawing shown in Fig. 5 is typical of a zero value while that shown in Fig. 4 is typical of a maximum score.

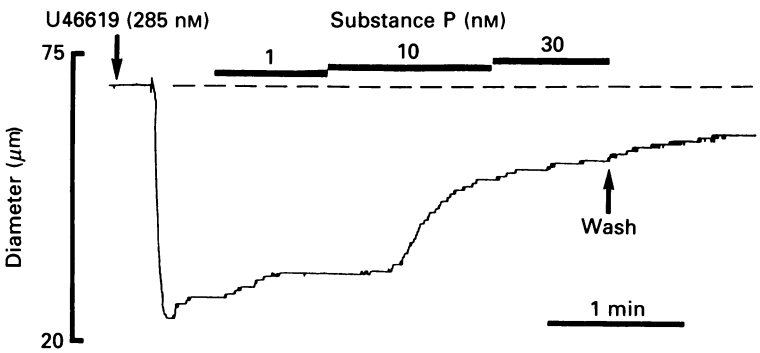

Fig. 7. SP-mediated vasodilatation of submucosal arteriole. Recording of outside diameter obtained from a normal preparation. SP was added to the superfusion fluid for the durations indicated by the bars above the trace; these concentrations of SP produced a sustained, dose-dependent relaxation of the blood vessel. The maximum dilatation to SP (82% of pre-constricted diameter) was the same as the maximum dilatation produced by muscarine (85%) or nitroprusside (83%) in this vessel.

not changed in the presence of tetrodotoxin (2 μM, $n = 3$), or the muscarinic receptor antagonists, pirenzepine (2–20 μM, $n = 6$), 4-DAMP (2 μM, $n = 6$) and AFDX-116 (2–20 μM, $n = 4$)

The SP antagonists, spantide, [APhTTL]SP and [APTTL]SP, were effective in

inhibiting the dilatations produced by applied SP (Fig. 8). [APhTTL]SP shifted the SP concentration–dilatation curve to the right in a parallel and competitive manner (Fig. 8*B*); Schild analysis of this data showed a linear relationship with a slope of $1{\cdot}2 \pm 0{\cdot}3$ and an apparent dissociation equilibrium constant for this antagonist of 170 ± 24 nM ($n = 5$). [APTTL]SP also shifted the curves to the right but at concentrations greater than 300 nM the maximum response to SP was reduced; i.e. [APTTL]SP acted as a non-competitive inhibitor of the SP vasodilatation (Fig. 8*C*).

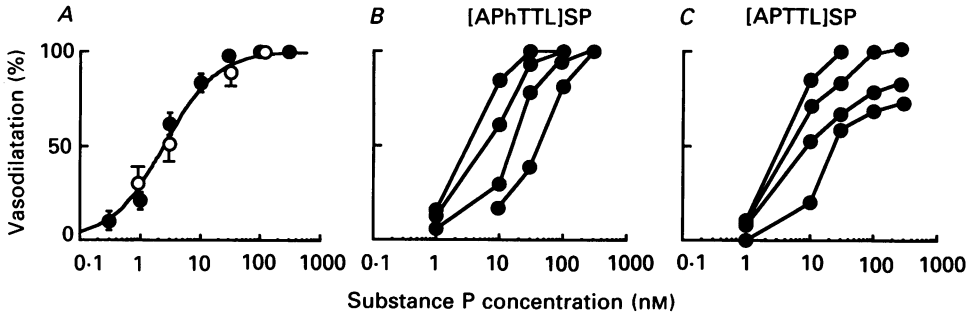

Fig. 8. Characterization of SP-mediated vasodilatation in submucosal arterioles. *A*, concentration–dilatation curves produced by application of SP in normal (●) and extrinsically denervated (○) preparations. EC_{50} value was 2·5 nM in both cases; $n = 6$ for all points from normal arterioles, $n = 4$ for all points from denervated preparations. Curve is drawn through points obtained from experiments on normal preparations and represents the best fit to a sigmoid curve; this curve yielded a 'Hill' slope of 1·002. *B*, competitive inhibition of the SP vasodilatation by the SP receptor antagonist [APhTTL]SP. Each point is the mean ± S.E.M. from four to six experiments carried out in normal preparations, the antagonist concentration was 0, 0·1, 0·3 and 1 μM respectively (leftmost to rightmost curve). *C*, non-competitive inhibition of the SP dilatation by the SP antagonist [APTTL]SP; experiments were carried out and results expressed in an identical manner to that described for *B* ($n = 4$ for each point), concentrations of antagonist were 0, 0·1, 0·3 and 1 μM left to right.

VIP and CGRP

The peptide VIP (1–600 nM) dilated 15/36 arterioles from normal and no arterioles (0/6) from extrinsically denervated preparations. The dilatation was inhibited by pirenzepine (2 μM) or 4-DAMP (200 nM) and it was enhanced by $39 \pm 3\%$ ($n = 4$) when the anticholinesterase, eserine, was applied. We therefore attributed the dilatation by VIP as being due primarily to VIP-evoked depolarization of cholinergic vasomotor neurones, as VIP is known to depolarize a proportion of submucosal neurones (Mihara, Katayama & Nishi, 1985; Surprenant, 1985). The peptide CGRP (1–300 nM) had no constrictor or dilator action on submucosal arterioles (nine normal and three denervated preparations).

DISCUSSION

This study's main finding is that a non-cholinergic vasodilator innervation to submucosal arterioles in the guinea-pig small intestine begins to function after

surgical removal of the extrinsic efferent and afferent nerve supply. Our results also make a strong case for SP as the transmitter of this non-cholinergic innervation. The evidence for SP being the non-cholinergic vasomotor transmitter can be summarized as follows: (1) SP application mimicked the neurogenic vasodilatation while other known vasodilators, VIP and CGRP, did not; (2) three SP antagonists, spantide, [APPTL]SP and [APhTTL]SP selectively inhibited both the SP-induced dilatation and the nerve-evoked dilatations over equivalent concentration ranges; (3) there was a significant correlation between projections of SP-containing nerve fibres from submucous ganglia to segments of arteriole which produced a non-cholinergic dilatation following stimulation of those ganglia.

In normal intestine, SP–CGRP-containing sensory fibres provide a dense innervation of submucosal arterioles; indeed, the sensory fibres are many times more numerous and make a more extensive perivascular plexus (Gibbins, Furness, Costa, MacIntyre, Hillyard & Girgis, 1985; Galligan *et al.* 1988) than the SP-containing fibres we observed after extrinsic denervation. Nevertheless, we have not been able to demonstrate a neurally mediated non-cholinergic vasodilatation in normal preparations. The SP-mediated vasodilatation was identical in normal and extrinsically denervated arterioles (Fig. 8); thus it is unlikely that extrinsic denervation alters SP receptor density or function in submucosal arterioles. It may be that electrical stimulation is insufficient to release SP from sensory fibres in this vascular bed but this seems somewhat unlikely because nervous release of SP and SP-mediated motor responses of the intestine have been demonstrated following mesenteric nerve stimulation. These motor responses are believed to be due to antidromic activation of SP-containing sensory fibres (see Bartho & Holzer, 1985 for review). A second possibility is that the probability of release of SP (per varicosity per action potential) is extremely low in sensory fibres associated with blood vessels such that the amount released is below threshold for vasodilatation. There is some precedence for noradrenaline (or ATP) being released with a very low probability from individual varicosities of sympathetic nerve fibres (Hirst & Neild, 1980; Cunnane & Stjarne, 1982; Hirst & Edwards, 1989). A third possibility may be that an inactive form of SP is released from these sensory fibres.

The development of the non-cholinergic, presumably SP-mediated, functional innervation may be due to fibre ingrowth from intrinsic SP-containing cell bodies or it may be due to an increased amount of (releasable) SP accumulating in existing nerve terminals. Our data cannot distinguish between these two possibilities. It is known that there are some SP-containing (without CGRP) fibres associated with normal submucosal arterioles that are largely obscured by the more numerous SP–CGRP-containing sensory fibres. These rare SP-containing fibres are more easily observed in tissues taken 3–5 days after denervation (Galligan *et al.* 1988). In addition, there are nerve cell bodies in submucous ganglia which contain SP, in addition to the acetylcholine-synthesizing enzyme, choline-acetyltransferase (ChAT; Furness, Costa & Keast, 1984). Perhaps these ChAT–SP-positive neurones release ACh preferentially in normal tissues but, following denervation, SP levels in the nerve terminals are increased so that the vasodilatory response is now a mixed cholinergic–non-cholinergic response.

In addition to the development of a functional SP-containing innervation of

submucosal arterioles, there was an increase in the number of VIP-containing nerve fibres associated with submucosal arterioles following denervation. Similar changes in submucosal VIP-containing nerve fibres following denervations have also been reported previously (Costa & Furness, 1983; Galligan *et al.* 1988). Although VIP has been demonstrated to be a potential mediator of neurogenic vasodilatations in some vascular beds (Bevan & Brayden, 1987), we could not demonstrate a direct action of VIP (either neurally released or exogenously applied) on guinea-pig submucosal arterioles in control or denervated tissues.

The physiological significance of the development of an additional vasodilator innervation subsequent to extrinsic denervation is unclear. It is the adrenergic vasoconstrictor, rather than the cholinergic vasodilator, innervation that is lost; yet there does not appear to be any obvious compensatory development of a vasoconstrictor response in submucosal arterioles following sympathectomy. That is, we detected neither a neurally mediated vasoconstriction nor an altered sensitivity to adrenergic (α_1) agonists. However, other possible alterations in submucosal arterioles following sympathectomy need to be examined; particularly in view of the strong arguments favouring ATP as the neurotransmitter responsible for the phasic contractions associated with sympathetic nerve stimulation (Burnstock & Griffith, 1988), it will be important to determine whether or not ATP-induced vasoconstriction changes.

The apparently full restoration of intestinal function following extrinsic denervation has fascinated investigators since the original experiments of Bayliss & Starling (1899) and Cannon (1906), not least because it is a rare example of so complete a functional recovery being achieved in adult mammals. This study adds to the growing body of evidence (see Furness & Costa, 1987) pointing to a precise reorganization of the neural circuitry among enteric neurones and their effectors (i.e. visceral smooth muscle, intestinal mucosa and intestinal vasculature) being directly responsible for such adaptation.

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