Journal of Physiology (1990), **420**, *pp.* 247–265
With 8 figures

Printed in Great Britain

VASODILATATION OF ARTERIOLES BY ACETYLCHOLINE RELEASED FROM SINGLE NEURONES IN THE GUINEA-PIG SUBMUCOSAL PLEXUS

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(*Received 31 March 1989*)

SUMMARY

1. The nervous control of arterioles in the guinea-pig submucosal plexus was studied. Outside diameters of arterioles were recorded using a video-monitoring system. Changes in arteriolar diameter in response to electrical stimulation of single neurones or ganglia in the plexus were measured.

2. When the arteriole was pre-constricted with the prostaglandin analogue U46619 or with phenylephrine, electrical stimulation (2–20 Hz, 10 s) of a ganglion dilated the blood vessel. This vasodilatation was abolished by tetrodotoxin or by cutting the fine nerve strands running between the ganglion and the arteriole.

3. The vasodilatations caused by ganglionic stimulation were blocked by the muscarinic antagonists atropine, pirenzepine, (11[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-)-one (AFDX-116), 4-diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP) and hexahydrosilodifenidol (HSDF). IC_{50} values for the inhibition of nerve-evoked vasodilatation by pirenzepine, AFDX-116 and HSDF were 500 nM, 4 μM and 25 nM respectively. Physostigmine (1 μM) increased the dilatation by 90%.

4. Muscarine dilated all submucosal arterioles; the concentration causing half-maximum effects was 200 nM. Muscarinic vasodilatations were inhibited by pirenzepine, AFDX-116, and HSDF in a competitive manner; dissociation equilibrium constants determined by Schild analyses were 125 nM, 1·3 μM and 4 nM respectively.

5. Gossypol, an irreversible inhibitor of the production of endothelium-derived relaxing factor (EDRF), did not reduce the vasodilatation produced by either ganglionic stimulation or muscarine in submucosal arterioles.

6. Intracellular recordings were made from submucosal neurones and action potentials were elicited by depolarizing current pulses (10 ms duration, 10 Hz/10 s). In seven neurones vasodilatation was associated with intracellularly evoked action potentials; this vasodilatation was blocked by pirenzepine. Cell bodies of reidentified

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vasodilator neurones were subsequently shown to contain immunoreactive choline acetyltransferase.

7. These results show that cholinergic neurones in the submucosal plexus project to submucosal arterioles and that they release acetylcholine onto muscarinic receptors to produce vasodilatation. The muscarinic receptor activated by nerve-released acetylcholine is the M_3 subtype and its location appears to be on the vascular smooth muscle rather than the endothelium.

INTRODUCTION

Sir Henry Dale (1913) demonstrated the presence of vasodilator innervation of the peripheral vasculature but identification of the origins of dilator nerves as well as the transmitter substance(s) mediating neurogenic vasodilatations has not been forthcoming (Bevan & Brayden, 1987; Burnstock & Griffith, 1988). Thus, while there are many examples of both atropine-sensitive and atropine-insensitive vasodilatations resulting from sympathetic, parasympathetic or reflex-initiated stimulation (Bülbring & Burn, 1936; Biber, Lundgren & Svanvik, 1971; Sjoqvist, 1983), two persistent barriers have hindered investigations of neurogenic vasodilatations: the inability to identify the cells of origin of the innervating fibres and the difficulty in obtaining a neurogenic cholinergic vasodilatation in isolation from non-cholinergic dilatations. Indeed, much evidence has accumulated for the co-existence and co-release of the potent vasodilators acetylcholine (ACh) and the vasoactive intestinal polypeptide (VIP) (Lundberg, Anggard, Fahrenkrug, Hokfelt & Mutt, 1980; Lundberg, 1981; Bevan & Brayden, 1987; Burnstock & Griffith, 1988).

The neurones of the submucosal plexus of the guinea-pig small intestine appear to contain either VIP or ACh but not both transmitters (Furness, Costa & Eckenstein, 1983; Furness, Costa & Keast, 1984; Furness & Costa, 1987). If any of these neurones innervate the arterioles which are present in the same plexus of the submucosa they would provide a unique opportunity to separate clearly the effects of these two transmitters by exciting individual cells. Submucosal arterioles are the final resistance vessels of the splanchnic circulation; identification of functional vasodilator innervation to these vessels would be of considerable physiological interest, as changes in blood flow in the splanchnic beds are a major determinant of total peripheral resistance in the systemic circulation. Thus, our initial aim was to determine whether stimulation of submucosal neurones would produce a physiological response in the arterioles with the expectation that the response would be a vasodilatation. We did discover such a response and this report describes the results from experiments in which we sought to characterize the neurogenic vasodilatation in submucosal arterioles. A preliminary report of some of these results has been presented in abstract form (Shen, Neild, Surprenant & Galligan, 1989).

METHODS

Submucosal plexus preparations were obtained from the small intestine of guinea-pigs; methods of tissue preparation, intracellular recording from submucosal neurones, focal extracellular stimulation of ganglia and drug applications were as detailed previously (Surprenant, 1984; Surprenant & Williams, 1987). Physiological saline solutions had the following composition (mM):

NaCl, 126; NaH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5; KCl, 5; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2 and 5% CO_2. Temperature was maintained at 34–36 °C. A fast flow rate (10–15 ml/min; bath volume 0·5 ml) was used because we found this procedure largely prevented tachyphylaxis of submucosal arterioles to vasoconstrictor substances which otherwise occurred. In some experiments the rat tail artery was used; tail arteries were obtained as described previously (Neild, 1987). In other experiments the prearteriolar mother vessel to the submucosal arterioles was used; this is the distal mesenteric artery found in the mesenteric arcade just before it dips into the intestinal wall proper.

Outside diameter of blood vessels was monitored using the Diamtrak system which has been described in detail (Neild, 1989). Briefly, microscope focus was adjusted until the outer edge of the arteriole was defined by a dark line and the TV camera was rotated until the arteriole was horizontal in the image. Television images of the arteriole were digitized using an Imaging Technology PCVisionPlus frame-grabber board in an IBM PC/AT computer. Using a mouse-controlled screen cursor, areas were then chosen for analysis such that one area would always include the top edge of the arteriole and the other area the bottom edge, and the edges of the arteriole were the darkest horizontal lines in these areas. A directional line detection procedure was applied to the selected areas of the image to locate the edges of the arteriole and the distance between the edges calculated. The result was converted to an analog signal which was displayed on a conventional chart recorder. The resolution of the system was equal to the distance between lines of the TV image; at the magnifications used this was less than 1 μm. The measurement rate depended on the total area of image analysed, and was in the range of 10–20 per second.

Extrinsic denervation alone or in combination with myectomy was accomplished as described in detail previously (Furness & Costa, 1978, 1987; Galligan, Costa & Furness, 1988); all denervations were performed under etorphine and Nembutal anaesthesia. Extrinsic denervation was confirmed using the glyoxylic acid fluorescence method (Furness & Costa, 1975); only those tissues in which no fluorescent fibres were present after surgery were used in this study. Myectomized preparations were used only if no longitudinal muscle was present over the circumference of the intestine (Costa & Furness, 1983; Bornstein, Furness & Costa, 1987).

Immunohistochemical localization of choline acetyltransferase (ChAT) was performed using the ChAT antibody and peroxidase–antiperoxidase (PAP) method described by Eckenstein, Baughman & Quinn (1988). ChAT-containing cell bodies were readily observed using this method; fibre projections could not be detected. We found that immunofluorescent methods for localization of ChAT (Furness, Costa & Eckenstein, 1983) did not label cell bodies as efficiently as the PAP method; it also did not allow detection of ChAT in fibres. Immunohistochemical localization of VIP was performed as described previously (Galligan, Costa & Furness, 1988); the antibody, VIP 7913, was provided by Dr J. H. Walsh.

Several methods have been used to remove endothelia from vessels too small to be amenable to direct mechanical disruption, such as cannulating the larger parent vessel and flushing or perfusing the attendent vascular branches with distilled water, detergents or collagenase solutions (Samata, Kimura, Satoh & Watanabe, 1986; Tesfamariam & Halpern, 1988; Furchgott, Carvalho, Khan & Matsunaga, 1987). Each of these methods, as well as many variants of these methods, were applied to the submucosal arteriolar network. All such manipulations invariably damaged the vascular smooth muscle in addition to any possible coincident effects on the endothelium; damage was manifest as an irreversible vasoconstriction which was not altered by vasodilator substances such as nifedipine (1 μM) or nitroprusside (1–20 μM), a significantly depolarized resting potential of the vascular smooth muscle measured using intracellular microelectrodes (−35 to −50 mV *vs.* −70 mV in normal arteriolar muscle), and little or no response to agonists such as prostaglandins, phenylephrine, muscarine or substance P.

Vasodilatations produced by nerve stimulation and pressure-ejection applications of agonists were quantified by measuring the 90% area under the curve of the vasodilatory response (e.g. Figs 2*C*, 3 and 6). All means are expressed with S.E. of the mean; tests of significance were performed using Student's *t* test. Dissociation equilibrium constants (K_D) for antagonists were estimated in the following manner; data from all experiments performed with a single antagonist were pooled and averaged dose–response curves obtained (e.g. Fig. 6*B*); these results were used to obtain K_D values (e.g. Fig. 6*C*) using the method of Arunlakshana & Schild (1959). Cumulative dose–response relations were obtained in all experiments and in any one experiment only two concentrations of

antagonist were used. Pressure-ejection pipettes were filled with 500 μM-nitroprusside or muscarine; pressure application was a single 300 ms duration pulse (10 lbf/in^2; 68 kPa) in all experiments.

The following drugs were used: DL-muscarine chloride, (−)scopolamine HCl, atropine, 1-phenylephrine, gossypol, eserine, sodium nitroprusside and tetrodotoxin (all from Sigma); (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, guanethidine (from CIBA), hexahydrosilodifenidol (synthesized and provided by G. Lambrecht, University of Frankfurt, FRG), 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin F2$_\alpha$ (U46619; Upjohn) and 4-diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP; Research Biochemicals Inc.).

RESULTS

Arteriolar responses to ganglionic stimulation

Constrictions

The resting diameters (outside diameter) of submucosal arterioles examined in these experiments ranged from 20 to 85 μm. When a focal stimulating electrode was placed on the surface of a ganglionic node from which fibre strands could be seen projecting toward an arteriole (Fig. 1*A*), electrical stimulation (1–20 Hz, 10 s) produced no response, or a small vasoconstriction in the blood vessels. This vasoconstrictor response was due to stimulation of the extrinsic sympathetic fibres which run through submucosal ganglia because it was not seen after surgical sympathectomy ($n = 8$) nor after superfusion with guanethidine (10 μM). Therefore, in subsequent experiments guanethidine was added to the bathing fluid if initial ganglionic stimulation resulted in constriction of the relaxed vessel.

Dilatations

When the arteriole was pre-constricted by superfusing the preparation with the thromboxane mimetic, U46619 (100–300 nM), or with the α_1-adrenoceptor agonist, phenylephrine (2–6 μM), ganglionic stimulation produced a vasodilatation (Figs 1*B–D* and 2*A*). Vasodilatation was observed during stimulation of 70 of 109 ganglia. We used phenylephrine to pre-constrict submucosal arterioles in the majority of experiments because no presynaptic or postsynaptic α_1-mediated responses can be detected in submucosal neurones (A. Surprenant, personal observations). A concentration of phenylephrine (2 or 6 μM) which produced approximately 80% of the maximum possible vasoconstriction (i.e. occlusion of the lumen) was used as the standard protocol. Ganglionic stimulation for 10 s produced a frequency-dependent vasodilatation with the peak response occurring at 10 Hz (Fig. 2*B*); dilatations in response to maximum stimulation (10 Hz/10 s) ranged from 15–100% of the pre-

Fig. 1. Photographs of the isolated submucous plexus showing arrangement of ganglion and arteriole; inset in *A* shows this arrangement diagrammatically. In *B* a focal stimulating electrode is brought into contact with the ganglia, arrows indicate outside diameter of the pre-constricted, unstimulated vessel (distance between arrows = 64 μm; same magnification in *B–E*). The preparation was then continuously superfused with 2 μM-phenylephrine (*C*). Photograph in *D* was taken approximately 1 s after cessation of focal stimulation (10 Hz for 10 s); the arteriole had relaxed to 82% of its pre-constricted diameter. Photograph in *E* was taken 35 s after cessation of the stimulation, by which time the vessel had reconstricted to the same diameter as in *C*.

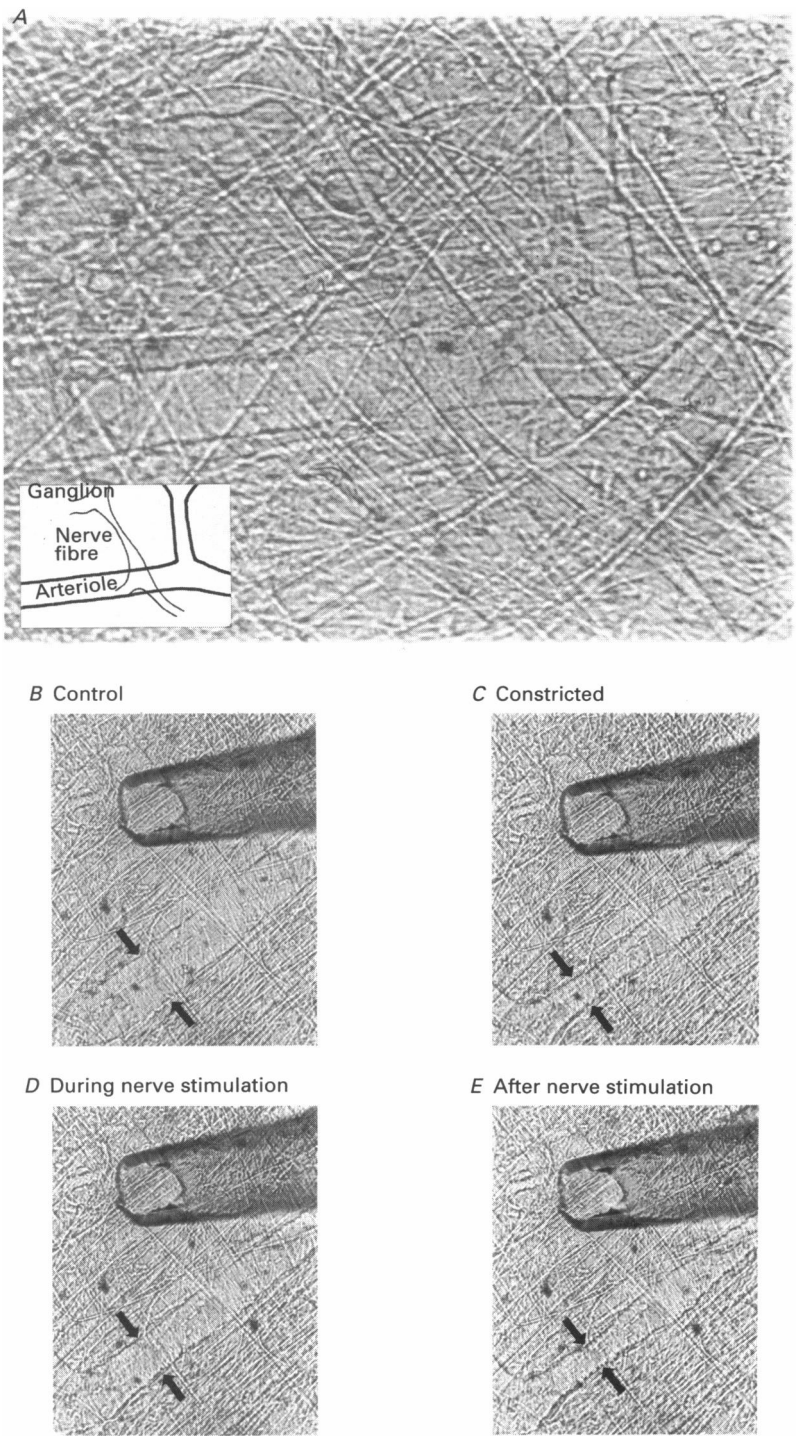

Fig. 1. For legend see facing page.

constricted diameter. Dilatation began 1–2 s after onset of the stimulation and outlasted the stimulation by 25–45 s (Fig. 2*A*). Vasodilatation evoked by repeated ganglionic stimulation at intervals of 15–20 min was remarkably consistent (within ±5%, Fig. 3*A–C*) for periods up to 4 h.

The neurogenic origin of these stimulation-evoked dilatations was established by three findings: (1) tetrodotoxin (200 nM) reversibly abolished the response ($n = 4$; Fig. 3*A*), (2) when the jagged edge of a broken microelectrode was used to cut the strands which projected from the stimulated ganglion towards the monitored blood vessel the response was abolished ($n = 3$), and (3) response could be elicited only when the focal stimulating electrode was placed directly on a ganglion. Interestingly, we were not able to elicit a vasodilatation by placing the stimulating electrode on the arteriole itself although this arrangement was always effective in eliciting a sympathetically mediated vasoconstriction of the relaxed vessel.

Muscarinic receptor antagonists blocked the vasodilatation produced by ganglionic stimulation in all experiments performed on preparations obtained from normal animals ($n = 19$; Figs 3*B* and 4); 200 nM-scopolamine or 4-DAMP abolished the vasodilatation produced by maximum stimulation (10 Hz/10 s) in all preparations. The anticholinesterase, physostigmine (1 μM) increased the magnitude of the vasodilatation produced by maximum stimulation by 88±9·6% ($n = 5$), further supporting its cholinergic nature.

The ability of the selective muscarinic antagonists pirenzepine, AFDX-116 and HSDF to inhibit stimulation-evoked vasodilatation was examined in a total of twenty-five arterioles. Each of these antagonists produced a concentration-dependent inhibition of the neurally elicited vasodilator response; IC_{50} values for HSDF, pirenzepine and AFDX-116 were 25, 500 and 4000 nM respectively (Fig. 4).

It was consistently observed that the vasoconstriction in response to either phenylephrine or U46619 was larger in the presence of any of the muscarinic antagonists used in these experiments. The increased magnitude of the agonist-induced vasoconstriction in the presence of muscarinic antagonists varied greatly among preparations, ranging from a 2 to a 36% increase (9±1·2%, $n = 27$). This value was significant ($P = 0·001$) when compared to consecutive vasoconstrictor responses obtained in the absence of any muscarinic antagonist (7±2% *decrease* in the response, $n = 21$). We have not studied this action of muscarinic receptor antagonists further but these results are quite suggestive of an on-going, basal level of muscarinic receptor activation by endogenous ACh.

Characterization of the muscarinic vasodilatation

The receptor type

In order to determine which muscarinic receptor subtype was being activated by the neurally released acetylcholine, we constructed concentration–response curves for the vasodilatation produced by steady-state superfusion with muscarine in the absence and then presence of muscarinic receptor antagonists (Fig. 5). Muscarine produced a concentration-dependent vasodilatation in all submucosal arterioles tested ($n = 10$); muscarine EC_{50} value was 200 nM (Fig. 6*A*). The muscarinic antagonists pirenzepine, HSDF and AFDX-116 each produced a rightwards, parallel shift in the muscarine concentration–dilatation curve; results obtained with pirenzepine are shown in Fig. 6*B*. Effects of each of these antagonists were fully

reversible within 30 min of washing from the bath. Schild plots determined from data obtained with each antagonist are shown in Fig. 6*C*. These yielded straight lines with slopes not differing significantly from unity; such results strongly imply that a single receptor type is being acted upon. Dissociation equilibrium constants (K_D) determined from these lines for HSDF, pirenzepine and AFDX-116 were 3, 125 and 1300 nM respectively (Fig. 6*C*). These values are very similar to those obtained from receptor binding data in exocrine gland preparations for the M_3 receptor subtype (Ladinsky, Giraldo, Monferini, Schiavi, Vigano, Deconti, Micheletti & Hammer, 1988; Bonner, 1989).

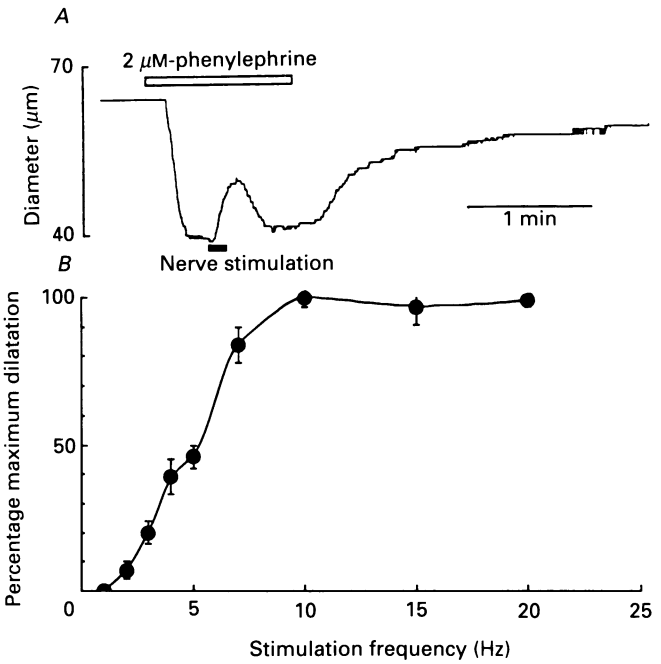

Fig. 2. Vasodilatation of submucosal arteriole recorded during focal stimulation of submucosal ganglia. *A*, trace of outside diameter of submucosal arteriolar segment; phenylephrine (2 μM) was superfused for the duration indicated by the open bar and a ganglion was stimulated (10 Hz/10 s) at the time indicated by the filled bar. *B*, vasodilatation elicited by 10 s stimulation periods at frequencies 1–20 Hz; threshold for initiation of vasodilatation was 2–3 Hz in all experiments. Results are expressed as a percentage of maximum dilatation recorded in each experiment; $n = 6$–8 for each point.

Site of action

Muscarinic vasodilatations in the vast majority of blood vessels appear to be mediated through the release of nitric oxide (Palmer, Ferrige & Moncada, 1987; Ignarro, Byrns, Buga, Wood & Chaudhuri, 1988) or a nitric oxide-like relaxing factor (EDRF) from endothelial cells (Furchgott & Zawadzki, 1980; Furchgott, 1984). Thus, abolition of the muscarinic relaxation after removal of the endothelium, or inactivation of EDRF synthesis and/or release, has been the primary criterion for determining whether acetylcholine exerts its actions through EDRF. As we were unable to physically remove the arteriolar endothelium without damaging the vessel

itself (see Methods), we examined the effects of gossypol, an irreversible inhibitor of EDRF (Busse & Forstermann, 1986; Alheid, Dudel & Forstermann, 1987), on the vasodilatation produced by muscarine and by nitroprusside, a vasorelaxant whose actions are enhanced after EDRF blockade (Shirasaki & Su, 1985; Alheid *et al.* 1987).

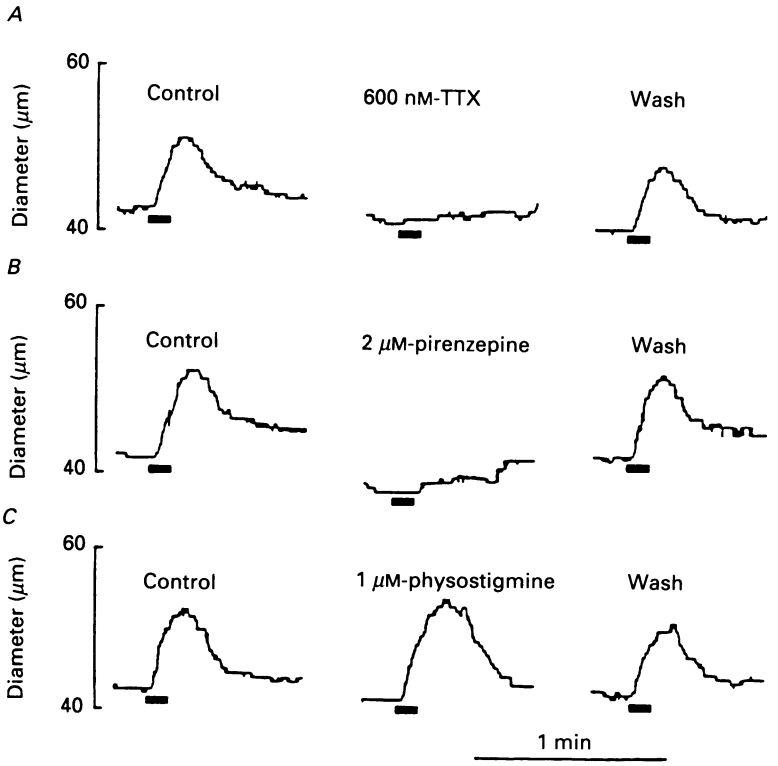

Fig. 3. On-line tracking of vessel diameter demonstrates the neurogenic cholinergic nature of stimulation-evoked vasodilatation. In all traces the filled bar indicates duration (10 s/10 Hz) of focal stimulation of submucosal ganglion. Phenylephrine was superfused as indicated in Fig. 2. TTX (*A*) and the muscarinic receptor antagonist, pirenzepine (PZP, *B*) blocked vasodilatation while physostigmine enhanced the response (*C*). All traces in *A–C* from the same arteriolar segment; 20 min wash-out period allowed between each stimulation, note consistency of response over many hours. Pre-constricted vessel diameter = 65 μm.

Gossypol, at concentrations up to 30 μM, did not alter the resting diameter of submucosal arterioles but concentrations greater than 5 μM inhibited or abolished the vasoconstriction elicited by either phenylephrine or U46619 ($n > 20$); 3 μM-gossypol had no significant effect on agonist-induced vasoconstrictions ($n = 17$). Applications of muscarine or nitroprusside at 15 min intervals produced reproducible vasodilatations ($\pm 10\%$) of the monitored arteriolar segment ($n = 20$). After treatment with 3 μM-gossypol the nitroprusside dilatation was increased by approximately 175% (Fig. 7*A* and *C*) and the muscarinic vasodilatation was also significantly increased (Fig. 7*B* and *C*). The gossypol-induced enhancement of the dilatations was not reversible within a 1 h wash-out period ($n = 3$, Fig. 7*A* and *B*).

Vasodilatations in response to ganglionic stimulation (10 Hz/10 s) were increased by $31 \pm 3\%$ ($n = 4$) after exposure to gossypol over responses elicited prior to treatment.

A similar series of experiments were performed on two larger vessels, vessels that would be expected to show endothelium-dependent responses to ACh, the tail artery

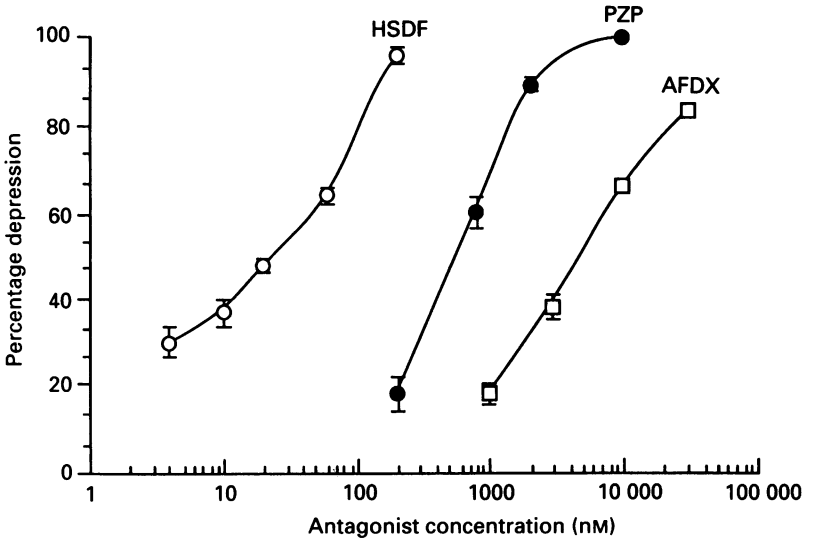

Fig. 4. Summary of depression of neurogenic vasodilatation in submucosal arterioles by muscarinic antagonists. Each point represents mean ± S.E.M. from four to six experiments. Effects of all concentrations of all antagonists were reversible within 20 min. The IC_{50} values for HSDF, pirenzepine (PZP) and AFDX-116 were 25, 500 and 4000 nM respectively.

(o.d. 750–800 μm) and the prearteriolar mesenteric artery (o.d. 250–700 μm) (Furchgott, 1984; Tesfamariam & Halpern, 1988). Arteries were pre-constricted with 20 μM-phenylephrine or 500 nM-U46614 and responses to superfusion with 20 μM-nitroprusside or 1 μM-muscarine were recorded. All arteries dilated in response to superfusion with either nitroprusside or muscarine ($n = 15$). Agonist-induced vasoconstrictions in the mesenteric artery, like those in its down-stream branches of the submucosal plexus, were inhibited or blocked by concentrations of gossypol greater than 10 μM and so 10 μM-gossypol was used in experiments on the mesenteric arteries.

Results obtained from experiments on the tail artery are shown in Fig. 7*C*; after gossypol treatment (30 μM) the nitroprusside vasodilatation was increased by 130% but the muscarinic vasodilatations were decreased by 72%. Lower concentrations of gossypol (3–5 μM) reduced the muscarinic dilatation by $42 \pm 6\%$ ($n = 4$) although these concentrations did not significantly increase the nitroprusside dilatation ($n = 4$). Qualitatively similar results were obtained from the mesenteric artery in which the muscarinic vasodilatation was inhibited by greater than 50% after exposure to gossypol (10 μM, $n = 5$).

Identification of the cholinergic vasodilator neurones

There are three possible sources of cholinergic innervation to submucosal arterioles: extrinsic vagal neurones, myenteric cholinergic neurones and the

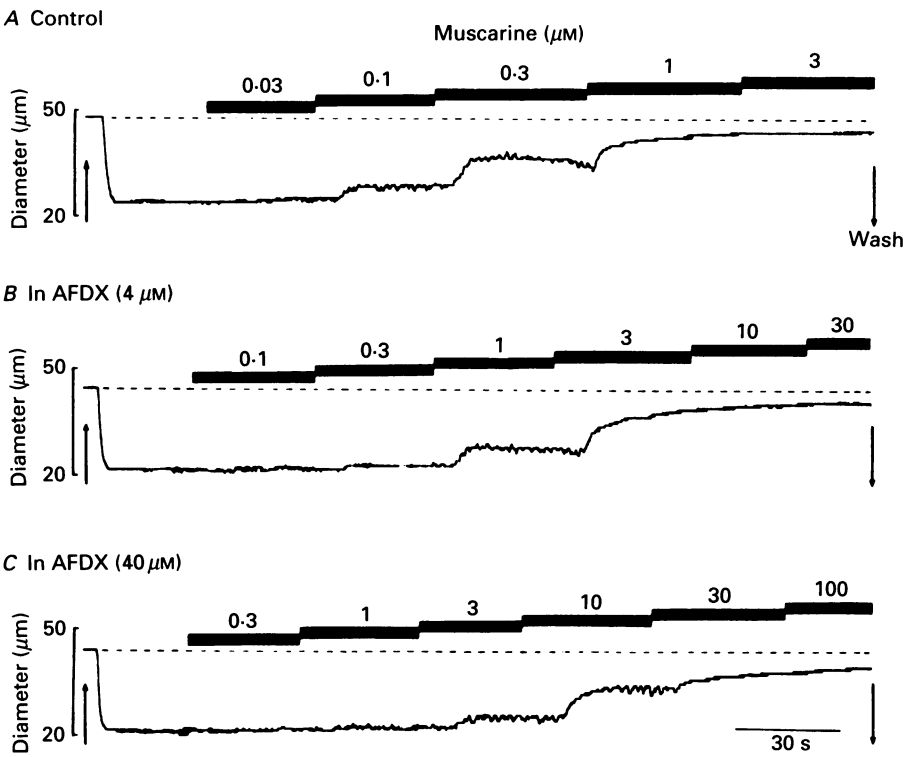

Fig. 5. Recordings of outside diameter of submucosal arteriole before (*A*) and during exposure to two concentrations of the muscarinic receptor antagonist, AFDX-116 (*B* and *C*); all recordings were obtained from the same arteriolar segment. Arteriole was pre-constricted with 285 nM-U46619 and increasing concentrations of muscarine were added to the perfusion solution as indicated by the filled bars above each trace; numbers refer to total muscarine concentration (μM).

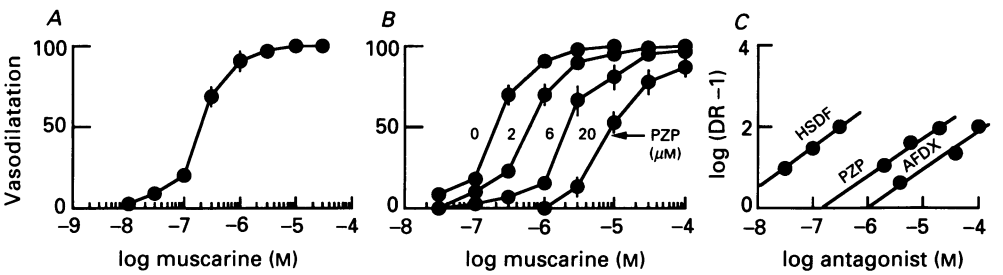

Fig. 6. Pharmacological characterization of muscarine-induced vasodilatation in submucosal arteriole. *A*, vasodilatation, expressed as percentage of maximum dilatation, in response to superfusion with muscarine; points are mean ± S.E.M, $n = 8$. *B*, pirenzepine (PZP) inhibition of muscarinic vasodilatation; each point is the mean ± S.E.M. from three to four experiments. *C*, Schild plots from data obtained as shown in *B*; log of dose ratio DR − 1 is plotted as a function of the log of the antagonist concentration. The x intercepts, or pA_2 values, for HSDF, PZP and AFDX-116 were −8·47, −6·9 and −5·9 respectively. Results shown for HSDF were obtained from three experiments, those for AFDX-116 from five experiments.

submucosal cholinergic neurones (Bornstein *et al.* 1987; Furness & Costa, 1987). The former two sources can be eliminated by combining extrinsic denervation with myectomy (Furness & Costa, 1987). We performed this surgical procedure on four guinea-pigs and confirmed the completeness of the denervations in all preparations

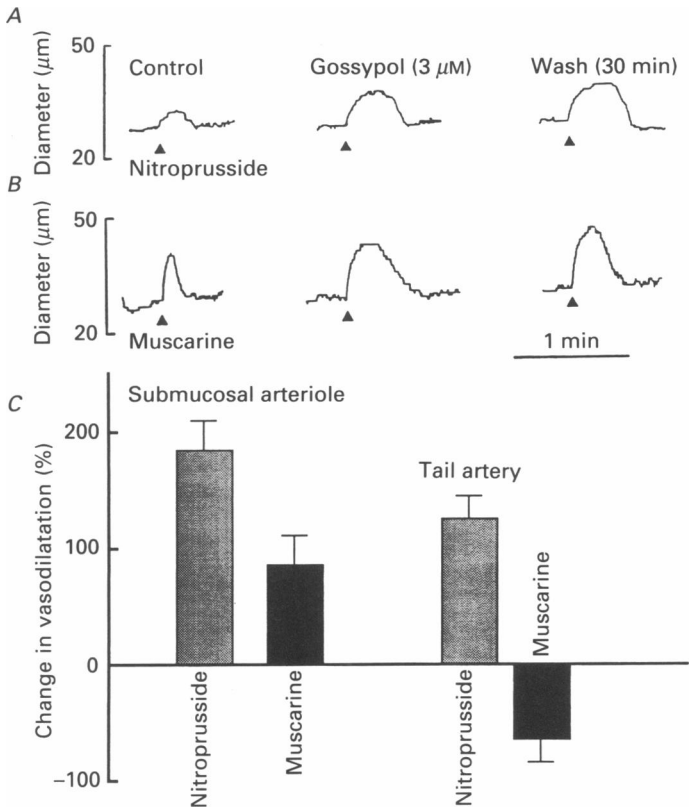

Fig. 7. Gossypol enhances both nitroprusside (*A*) and muscarine (*B*) vasodilatations in submucosal arterioles but depresses muscarinic vasodilatation of rat tail artery (*C*). Dilatations in response to pressure-ejection application of nitroprusside or muscarine are shown before, 10 min after addition of 3 μM-gossypol to the perfusion fluid and 30 min after wash-out of gossypol. U46619 (285 nM) was used to pre-constrict the arteriole; pre-constricted outside diameter = 68 μm. *C*, summary of the effects of gossypol on both submucosal arteriole and rat tail artery. Results are expressed as percentage change from response recorded in control solution, $n = 10$ for experiments performed on submucosal arterioles; results with nitroprusside on tail artery are from four experiments and those with muscarine are from eleven experiments.

(see Methods). All experiments were performed 6–8 days after surgery. The vasodilatation elicited by stimulating a ganglion was increased rather than decreased in lesioned preparations. For equal-sized vessels (o.d. 55–60 μm) the relaxation (as percentage of pre-constricted diameter) to maximum stimulation was $52 \pm 5\%$ ($n = 3$) in operated, and $38 \pm 7\%$ ($n = 6$) in control, preparations. Additionally, the proportion of ganglia from which focal stimulation produced a dilatation in the monitored arteriole was greater in lesioned preparations; 90% (28/31) of ganglia stimulated produced vasodilatations in lesioned preparations.

Results from the lesioning experiments strongly suggested that the origin of the vasodilator neurones were the submucosal cholinergic neurones. Therefore, we next made intracellular recordings from individual submucosal neurones in ganglia in which focal stimulation dilated the monitored arteriolar segment. Depolarizing

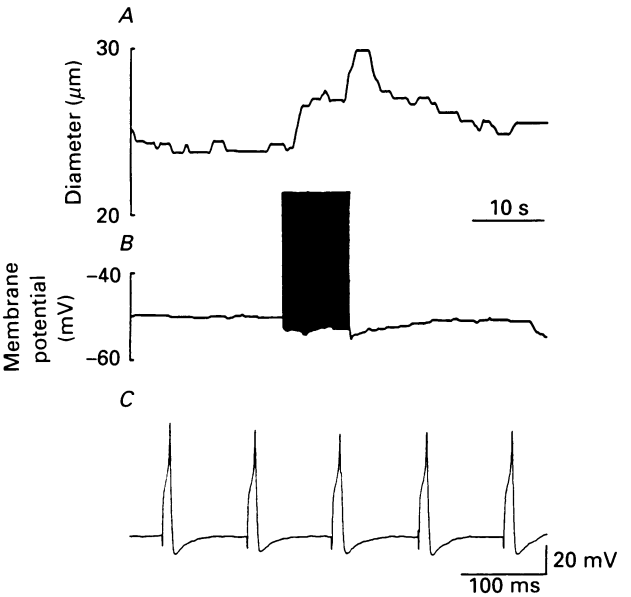

Fig. 8. Simultaneous measurement of arteriolar diameter (*A*) and membrane potential of a submucosal neurone (*B* and *C*). Depolarizing current pulses evoked action potentials (*B*, the first five of which are shown on faster time scale in *C*) which preceded development of arteriolar vasodilatation. Pre-constricted arteriolar diameter = 45 μm.

current pulses (10 ms duration, 10 Hz for 10 s) passed through the intracellular recording electrode evoked action potentials in all neurones; in seventeen of twenty-four trials this was not associated with any change in arteriolar diameter. In seven trials, vasodilatation began about 1 s after initiation of action potentials, and followed the same time course as did the vasodilatation induced by focal extracellular stimulation (Fig. 8). Action potential-induced vasodilatations were blocked by 2 μM-pirenzepine. In the experiment shown in Fig. 8 the magnitude of the vasodilatation produced by intracellular stimulation of the single neurone was the same as that produced by focal stimulation of the ganglion surface. The vasodilatation elicited by intracellular stimulation in the other six cells ranged from 40–100% of the response produced by focal stimulation. It should be noted in this regard that previous experiments in which simultaneous intracellular recordings were made from pairs of submucosal neurones within the same node failed to provide any evidence for electrical coupling between neurones (total of sixty-four pairs examined; Y. Katayama and A. Surprenant, unpublished observations); thus, it is extremely unlikely that electrotonic spread of the depolarization into adjacent cells occurred.

A submucosal ganglion consists of about five to fifteen neurones abutting each other in a monolayer, an arrangement which allows a neurone to be identified and impaled under direct visual control and then to be re-identified after processing for

immunohistochemistry. The peroxidase–antiperoxidase method for immunohistochemical localization of ChAT (Eckenstein *et al.* 1988) was performed on three of the preparations in which an identified neurone produced the vasodilatory response; these three cells all contained immunoreactive ChAT. VIP immunohistochemistry was performed on the remaining four preparations in which intracellular stimulation of a neurone caused a vasodilatation. One of these neurones could not be relocated but none of the other three contained VIP immunoreactivity. The corollary of this observation is that they all contained ACh (see Furness & Costa, 1987).

DISCUSSION

This study has demonstrated a vasodilator innervation to submucosal arterioles and directly identified both the origin of the dilator nerves and the transmitter released from these fibres that causes the vasodilatation. That is, the source of the dilator fibres was found to be the submucosal neurones because removal of extrinsic and myenteric sources of innervation did not alter the neurogenic vasodilatation and because of the unequivocal finding that intracellular stimulation of a single submucosal neurone elicited the arteriolar vasodilatation. The evidence that the vasodilator transmitter released from these nerves is acetylcholine is severalfold: all muscarinic receptor antagonists examined blocked the neurally mediated vasodilatation, exogenous application of muscarine mimicked the response and was blocked by the same concentrations of muscarinic antagonists that blocked the neurally evoked vasodilatation, the anticholinesterase inhibitor, physostigmine, increased and prolonged this response, and the cell bodies from which intracellular stimulation produced arteriolar vasodilatation contained ChAT immunoreactivity.

Results from this study bear directly on two distinct areas of physiology, vasodilator mechanisms and functions of the enteric nervous system.

The muscarinic vasodilatation in submucosal arterioles

It has been a nearly universal finding that peripheral mammalian arteries possessing an intact endothelium dilate in response to exogenously applied acetylcholine (Furchgott, 1984; Bevan & Brayden, 1987; Burnstock & Griffith, 1988). Nevertheless, convincing evidence for cholinergic vasodilator transmission exists only for guinea-pig uterine arteries and feline and rabbit cephalic and lingual arteries but there is little information as to the source of the cholinergic innervation to these arteries (Bell, 1969; Iwayama, Furness & Burnstock, 1970; Duckles, 1981; Bevan, Buga, Jope, Jope & Moritoki, 1982; Brayden & Large, 1986; Bevan & Brayden, 1987). The cholinergic innervation to submucosal arterioles demonstrated by this study should provide a well-defined system for further studies of muscarinic vasomotor functions.

One of the more intriguing aspects of this study was the finding that the muscarinic vasodilatation appeared to be endothelial cell independent. The ability of gossypol to block EDRF by inhibiting its release and/or synthesis has been documented in other arteries (Busse & Forstermann, 1986; Alheid *et al.* 1987); our results, in which gossypol selectively inhibited the muscarinic vasodilatation in two larger arteries, would tend to substantiate the use of gossypol in differentiating

between endothelial-dependent and independent mechanisms of vasodilatation. It is not known why nitrovasodilator responses are observed to be enhanced after either mechanical disruption of vascular endothelium (Shirasaki & Su, 1985) or treatments which interfere with EDRF actions (Alheid *et al.* 1987); however, the clear increase in the nitroprusside vasodilatation of submucosal arterioles after gossypol treatment further supports our conclusion that gossypol did effectively block the actions of EDRF in these vessels. Nevertheless, further studies using a greater variety of pharmacological manipulations to interfere with EDRF-related mechanisms will be required before we can consider this conclusion valid.

Whether acetylcholine released from perivascular nerve fibres does reach the intimal surface to activate muscarinic receptors on endothelium has not been directly established (Angus, Campbell, Cocks & Manderson, 1983; Cohen, Shepard & Vanhoutte, 1984). The muscarinic vasodilatation of rabbit lingual artery, one of the few vessels for which cholinergic neurogenic dilatation has been adequately established, also appears to be endothelial-cell independent (Brayden & Large, 1986; Bevan & Brayden, 1987). This finding, coupled with our results on submucosal arterioles, might suggest that acetylcholine mediates its vasodilatory actions directly through the smooth muscle in those blood vessels that possess a functional cholinergic innervation.

Molecular cloning techniques have resulted in the identification of at least five separate subtypes of the muscarinic receptor (Bonner, Buckley, Young & Brann, 1987; Peralta, Winslow, Peterson, Smith, Ashkenazi, Ramachandran, Schimerlik & Capon, 1987; Peralta, Winslow, Ashkenazi, Smith, Ramachandran & Capon, 1988). Muscarinic receptors mediating vasodilatation, whether through endothelial or smooth muscle, have previously been shown to belong to the original 'M_2-cardiac' subtype (Burnstock & Griffith, 1988); this subtype now comprises M_2, M_3 and M_4, with the M_2 and M_4 subtypes generally being associated with cardiac and visceral muscle as well as with presynaptically located receptors on neuronal tissue (Ladinsky *et al.* 1988; but see also Bonner, 1989 for comparative terminology) while the M_3 subtype is now associated with the muscarinic responses observed in exocrine gland cells (the original 'M_2-exocrine' subtype; Ladinsky *et al.* 1988; Peralta *et al.* 1988; Bonner, 1989). Few studies using selective muscarinic receptor antagonists that can distinguish between the three 'M_2' subtypes have been carried out on blood vessels. Our results provide rather conclusive evidence that it is the M_3 'exocrine' subtype of muscarinic receptor which is activated by endogenously released acetycholine in submucosal arterioles. Pharmacological characterizations of the subtype of muscarinic receptors present on other blood vessels, using the now-available selective antagonists, will establish whether the presence of M_3 muscarinic receptors is the rule or the exception in other vascular systems.

Our method for monitoring outside diameter of small vessels provides a straightforward means for studying physiological and pharmacological properties of small resistance vessels which hitherto have been inaccessible for *in vitro* experiments and, in particular, the small vessels (outside diameters $< 150\ \mu m$) which may be embedded in connective tissue (see Neild, 1989). As it is the resistance vessels that play the major role in changes of systemic blood pressure, the ability to routinely

track the diameter of small resistance arterioles should be helpful in the development of drugs or other therapies for the control of blood pressure.

A vasomotor role for submucosal neurones in the enteric nervous system

Whole animal studies have demonstrated that functional vasodilator innervation from autonomic neurones to blood vessels of the gastrointestinal tract is present (Dale, 1913; Bülbring & Burn, 1936; Hulten, 1969; Biber, Fara & Lundgren, 1973; Sjoqvist, 1983) but it has not been known whether this dilator innervation derived from extrinsic sympathetic/parasympathetic origins or from intrinsic enteric neurones. Our study has provided a direct demonstration that the vasodilator nerves to blood vessels of the guinea-pig small intestine are a population of cholinergic submucosal neurones. That submucosal neurones are involved in the control of water and electrolyte transport by the intestinal mucosa has been well established by studies carried out over the past decade (see reviews by Tapper, 1983; Hubel, 1985; Cooke, 1986; Keast, 1987). Submucosal neurones innervating gastrointestinal mucosa are secretomotor; that is, their activation promotes water and electrolyte secretion (Cooke, 1986; Keast, 1987). The additional and complementary vasodilatory role of submucosal neurones may provide for a simultaneous increase in blood flow to the intestinal mucosa in concert with its direct excitation of the mucosa. Many submucosal neurones injected with Lucifer Yellow dye show axons with one or more collaterals, collaterals that can be observed to cross over and/or run with arteriolar segments (Bornstein, Furness & Costa, 1987; K.-Z. Shen and A. Surprenant, personal observations); thus, the possibility exists that a single cholinergic submucosal neurone may provide a dual innervation to arteriole and intestinal mucosa. An analogous situation has been demonstrated in exocrine gland tissues where parasympathetic nerve stimulation causes both vasodilatation and fluid secretion; here, VIP and ACh co-exist but appear to be differentially released such that VIP primarily mediates the vasodilatation while ACh promotes fluid secretion (Lundberg *et al.* 1980; Lundberg, 1981).

Three subpopulations of cholinergic submucosal neurones in the guinea-pig small intestine can be differentiated based on patterns of co-localization of peptides with ChAT: (1) about 60% of ChAT-positive neurones co-localize with a family (CCK/CGRP/NPY/SS) of peptides, (2) about 15% are ChAT/SP positive and (3) another 25% of the ChAT-positive cells do not stain for any of the currently available peptide antibodies (see Furness & Costa, 1987). We have performed immunostaining with antibodies raised against neuropeptide Y (NPY) and SP on four of the submucosal neurones from which intracellular stimulation elicited vasodilatation and neither of these peptides was present (Galligan, Jiang, Shen & Surprenant, 1990). Further experiments of this kind, in which functionally identified vasodilator neurones are subjected to double-staining immunohistochemical analysis, will be required to establish whether the vasodilator neurones form a distinct subpopulation of cholinergic neurones. In any event, vasodilator innervation to the arterioles seems to represent a substantial number of the cholinergic neurones in view of our finding that about 65% of the ganglia we stimulated elicited vasodilatation. This proportion is probably an underestimate of the vasodilatory contribution of each ganglion

because the video-monitoring system tracked diameter in only one arteriolar segment of a widely branching tree at any one time. Indeed, we occasionally saw on the video screen a localized vasodilatation in a branch upstream or downstream to a non-responsive segment being quantitatively monitored. On the other hand, vasodilatation in response to a stimulation of a single neurone occasionally was equal to that produced by stimulation of the whole ganglion (each ganglion comprising approximately fifteen neurones of which half are cholinergic), suggesting that only one or two neurones per ganglion may be cholinergic vasomotor nerves.

We found no evidence for a non-cholinergic neurogenically mediated vasodilatation in the small intestine of normal guinea-pigs (but see Galligan *et al.* 1990). This finding is in contrast to results from a large body of work (*in vivo* and *in vitro*) performed on the cat colonic blood vessels which has led to the conclusion that a peptide, most likely vasoactive intestinal polypeptide (VIP), mediates a portion of the neurogenic vasodilatation in addition to the cholinergic component which is also present (Hulten, 1969; Biber, Fara & Lundgren, 1973; Sjoqvist, 1983). It may be that VIP and acetylcholine co-exist in nerves innervating the colonic vasculature, as they appear to in many other autonomic nerves (Lundberg *et al.* 1980; Lundberg, 1981), resulting in stimulation-evoked release of both vasodilators; submucosal neurones of the small intestine appear unusual in that VIP and cholinergic neurones form separate and mutually exclusive populations (Furness & Costa, 1987). Nevertheless, VIP-containing varicose nerve fibres course along submucosal arterioles, at least some of which appear to originate from submucosal neurones (Costa & Furness, 1983; Galligan *et al.* 1988), and it was somewhat surprising to have obtained no suggestion for a non-cholinergic component in normal animals as we were certain to have stimulated as many VIP neurones as cholinergic neurones in our experiments.

Conclusions

We have used a new video-monitoring system to continuously track outside diameter of small resistance vessels in the guinea-pig small intestine in order to determine whether a physiological response to stimulation of the submucosal neurones was present. Depolarization of individual neurones by intracellular microelectrodes, or stimulation of a small group of neurones by a focal external electrode, resulted in vasodilatation that was found to be due to the release of acetycholine acting on muscarinic M_3-type receptors, receptors which appear to be located on the vascular smooth muscle.

This work was supported by grants from the American Heart Association (86-1242) and US Department of Health and Human Services HL38940. M-M. Jiang provided invaluable technical assistance. We thank Dr J. J. Galligan for instructions and help with surgery and immunohistochemistry and Dr F. Eckenstein, who provided the ChAT antibody, for help with this immunostain.

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