Actions of vasodilator nerves on arteriolar smooth muscle and neurotransmitter release from sympathetic nerves in the guinea-pig small intestine

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1. Brief constrictions of arterioles of the isolated submucosa of the guinea-pig small intestine were evoked by stimulation of the perivascular sympathetic nerves. Prior stimulation of vasodilator neurones in the submucosal nerve plexus greatly reduced the constrictor response to sympathetic stimulation.

2. Vasodilator nerve stimulation reduced both the amplitude and rate of decay of the excitatory junction potential (EJP) evoked in the arteriolar smooth muscle by sympathetic nerve stimulation.

3. Computer simulation of the effect of membrane resistance changes on the EJP amplitude indicated that the change in amplitude could not be explained by the fall in membrane resistance alone, suggesting that vasodilator nerve activity reduced neurotransmitter release from the sympathetic nerves.

The blood flow to the intestine is normally highly variable, responding to the need for fluid secretion and absorption and the metabolic demands of the mucosa and muscle. It has long been known that intestinal blood flow is influenced by well-developed neural regulatory mechanisms within the enteric nervous system of the intestine itself, as well as by extrinsic nerves and hormones such as secretin, gastrin, and cholecystokinin. Food or bile acids in the lumen of the small intestine increase mesenteric blood flow by a mechanism that does not require the extrinsic nerve supply to the intestine, and reflex control of intestinal arteriole diameter can be demonstrated in isolated intestinal preparations (Vanner, Jiang & Surprenant, 1993). The action of cholera toxin on the small intestine also involves intestinal vasodilator neurones (Cassuto, Siewert, Jodal & Lundgren, 1983; Jiang, Kirchgessner, Gershon & Surprenant, 1993).

The arterioles of the small intestine are supplied with nerves which originate in the submucosal plexus of the enteric nervous system (Galligan, Costa & Furness, 1988; Brookes, Steele & Costa, 1991), and the vasodilator effects of these neurones can be demonstrated in isolated preparations of the submucosal plexus of the guinea-pig (Neild, Shen & Surprenant, 1990). Acetylcholine is a major vasodilator neurotransmitter in this system, but others are clearly involved. Vasoactive intestinal polypeptide (VIP), galanin, and dynorphin are present in some of the neurones and have vasodilator effects when applied to the vessels (Kotecha & Neild, 1995). In the rat VIP has been implicated in intrinsic vasodilator reflexes (Rozsa & Jacobson, 1989).

Our previous studies on guinea-pig intestinal arterioles have demonstrated that dilator nerves can reduce the constriction induced by drugs such as noradrenaline. However, this gives limited information about their physiological effectiveness in situations where the contractile state of the arteriolar muscle is determined by the level of sympathetic nerve activity. There is the potential for vasodilator nerves to regulate sympathetic neurotransmitter release; activation of prejunctional muscarinic receptors reduces neurotransmitter release from sympathetic nerves in the arteries (Komori & Suzuki, 1987; Fernandes, Alonso, Marin & Salaices, 1991) and the vas deferens (Eltze, 1988). In the experiments described below we sought evidence of an effect of submucosal vasodilator nerves on neurotransmitter release from sympathetic nerves.

METHODS

Guinea-pigs (Monash outbred strain) of either sex and weighing 200–300 g were killed by a heavy blow to the head followed by exsanguination, and a piece of ileum was removed. The ileum was slit open and pinned out mucosa uppermost, and the mucosa peeled off. A sheet of connective tissue containing the submucosal arterioles and nerve plexus was then separated from the circular muscle and pinned out in a small chamber with a transparent base. The sheet was superfused continuously with warmed oxygenated physiological saline, composition (mmol l^{-1}): Na^+, 146; K^+, 5; Ca^{2+}, 2·5; Mg^{2+}, 2; Cl^-, 134; HCO_3^-, 25; $H_2PO_4^-$, 1; glucose, 11; and was equilibrated with 95% O_2–5% CO_2.

The preparation was viewed with an inverted compound microscope equipped with a television camera, and arteriole diameter was monitored by computer analysis of the television images (Neild, 1989). This method measures average diameter over

a chosen region of arteriole less than 100 μm long. The computer marks the edges of the vessel on the television screen during the experiment, so that any transient errors in measurement can be identified.

Nerves were stimulated using a bevelled glass pipette of tip diameter 50–80 μm (Neild *et al.* 1990). The pipette was filled with the same physiological saline that was used to superfuse the preparation, and connected to the cathode of a conventional nerve stimulator (Grass S88 with SIU5A isolator). The anode of the stimulator was connected to an indifferent electrode in the recording chamber. A pulse duration of 0·1 ms was used. Pipettes were placed over a single submucosal ganglion for vasodilator nerve stimulation, or on the surface of the arteriole at least 1 mm central to the point at which diameter was monitored to stimulate perivascular nerves. U46619 was applied by pressure ejection from a micropipette, using a pulse duration of 1 s. This gave a brief local constriction whose size could be adjusted by slight changes in the pipette position.

Drugs used were: 9,11-dideoxy-9α, 11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619, Cayman Chemical Co., Ann Arbor, MI, USA), phentolamine mesylate (Regitine, Ciba), pirenzepine dihydrochloride (Sigma).

All means are given with the standard error of the mean and the number of observations. Each observation was made on a different arteriole; in some cases more than one arteriole was obtained from one animal. Experiments were carried out on arterioles with diameters in the range 52–95 μm. Changes in diameter are expressed as a percentage of the resting diameter. In previous experiments it was found that the maximum constriction in submucosal arterioles was 54·3 ± 1·4% ($n = 34$) of the resting diameter.

Intracellular recordings of membrane potential were made using conventional glass microelectrodes filled with 2 M KCl and with resistances in the range 100–200 MΩ. The recording was made within the region in which diameter was monitored or close to it, within 50 μm of one end of the region. For measurement of the amplitude and time constant of decay of excitatory junction potentials (EJPs) the membrane potential was recorded using an analogue tape recorder and replayed later to a computerized data analysis system.

Calculation of the effect of changes in membrane resistance on EJP amplitude

The EJP was simulated using eqn (A5) of Edwards, Hirst & Silinsky (1976). Their paper deals with synaptic potentials in nerve cells and assumes that the membrane is isopotential at all times. This assumption is also valid for arterioles when the EJP is considered. Although the smooth muscle syncytium is an extended structure in which potential gradients can exist, the sympathetic innervation is spread across the muscle and releases neurotransmitter to all regions virtually simultaneously when causing the EJP (Hirst & Neild, 1978).

It was assumed that the time course of the current causing the EJP could be described by the equation:

$$I(t) = At\mathrm{e}^{-\alpha t}, \quad (1)$$

where $I(t)$ is the current, A is a measure of current intensity, t is time, and α is the reciprocal of the time-to-peak of the current. This function with a value for α of 0·05 ms^{-1} gives a good approximation of the EJP current for the arterioles used in this study (Hirst & Neild, 1978; Finkel, Hirst & VanHelden, 1984).

The time course $V(t)$ of the EJP in an arteriole is given by:

$$V(t) = \frac{A}{C(1/\tau - \alpha)^2}[\{(1/\tau - \alpha)t - 1\}\mathrm{e}^{-\alpha t} + \mathrm{e}^{-t/\tau}] + E_{\mathrm{m}}, \quad (2)$$

where C is a measure of membrane capacitance, τ is the membrane time constant, and E_{m} is the resting membrane potential. The membrane resistance affects the EJP via its contribution to τ, which is the product of membrane resistance and capacitance. For our purposes explicit values for A and C do not need to be known; both can be assumed to remain the same throughout one experiment because we recorded from the same point (same C) and used the same stimulus parameters (same A). A/C therefore becomes an arbitrary scaling constant that was determined for each experiment.

The time constant of decay of the control EJP was measured by fitting a straight line to a semilogarithmic plot of the EJP decay. The decay time constant is a good estimate of τ (Hirst & Neild, 1978). Equation (1) was then used to calculate a simulated EJP of the same amplitude as the one recorded and determine a value for the scaling factor A/C. The time constant of the EJP after vasodilator nerve stimulation was then measured, and a simulated EJP calculated using this time constant and the same scaling factor. The amplitude of this simulated EJP was noted; it was the amplitude expected if the amount of neurotransmitter had not changed.

RESULTS

Vasodilator nerves reduce the constrictor response to sympathetic stimulation

Brief constrictions were evoked once every 50 s by stimulation of the perivascular nerves for 1 s at 10 Hz. In sixteen arterioles of mean diameter 77·5 ± 2·80 μm ($n = 16$) the constriction was on average 11·9 ± 0·69 μm, or 15·6 ± 1·02% of the resting diameter of the arteriole. The constriction was unaffected by 1 μM phentolamine. In five experiments the mean amplitude of the control constriction was 16·2 ± 1·64% and after 10 min in 1 μM phentolamine it was 17·4 ± 1·54%, which was not significantly different (Student's paired t test). This indicated that the constriction was not mediated by noradrenaline acting on α_1 receptors, and was probably caused by EJPs which summate to give an 'active response' in the smooth muscle (Hirst, 1977; Neild & Zelcer, 1982) as shown in Fig. 2.

The perivascular nerves that run along the arteriole include both sympathetic and sensory fibres, and both will have been stimulated in our experiments. Perivascular stimulation always gives constriction in this preparation, presumably via sympathetic nerves. Although sensory fibres can release both calcitonin gene-related peptide (CGRP) and vasodilator tachykinins, we have not recorded hyperpolarization due to stimulation of these nerves as observed by Meehan, Hottenstein & Kreulen (1991), perhaps because the resting membrane potential in the isolated arterioles is quite close to the K^+ equilibrium potential. We feel that the major effect must be mediated by the sympathetic nerves, as there was always a strong correlation between constriction and the active responses due to summed EJPs.

Table 1. Effect of stimulation of a submucosal ganglion at 10 Hz for 10 s on the amplitude of vasoconstriction caused by either perivascular nerve stimulation at 10 Hz for 1 s, or application of U46619 by pressure ejection from a micropipette for 1 s

	Prestimulation			Ganglion stimulation		
	Control	Phentolamine (1 μM)	Pirenzepine (2 μM)	Control	Pirenzepine (2 μM)	Number of arterioles
Perivascular	15·6 ± 1·02	—	—	4·98 ± 0·92*	—	16
stimulation	16·2 ± 1·64	17·4 ± 1·54	—	—	—	5
	13·3 ± 1·67	—	—	5·93 ± 2·38*	—	5
	—	—	13·4 ± 2·98	—	11·7 ± 2·79*	—
U46619	14·7 ± 1·31	—	—	6·82 ± 1·9*	—	4

Results show amplitude of constriction (mean ± S.E.M.) given as a percentage of resting diameter. An asterisk indicates that the value is significantly different (Student's paired t test, $P < 0·05$) from its respective control.

A nearby submucosal ganglion was stimulated at 10 Hz for 10 s. These parameters give a maximal dilator effect in arterioles that were pre-constricted by an exogenous vasoconstrictor substance such as phenylephrine (Neild *et al.* 1990), but have no effect on the diameter of an unstimulated arteriole because in these isolated preparations the arteriolar smooth muscle is completely relaxed (Neild & Kotecha, 1989). Ganglion stimulation reduced the constriction caused by perivascular stimulation to 4·98 ± 0·92%, significantly less than the control value of 15·6%.

Our previous work (Neild *et al.* 1990; Kotecha & Neild, 1995) had shown that the effects of these vasodilator nerves was reduced by the muscarinic antagonist pirenzepine. In five experiments a high concentration of pirenzepine (2 μM, sufficient to block all classes of muscarinic receptor; Eglen & Whiting, 1990) significantly reduced the effect of vasodilator nerve stimulation (Table 1). This confirmed our earlier findings that suggested a major role for acetylcholine as a vasodilator neurotransmitter. We did not attempt to analyse these results further as our previous work (Neild & Kotecha, 1995) showed that vasodilator responses were a heterogeneous population of cholinergic and non-cholinergic effects.

The present experiments also differ from previous work in the nature and timing of the vasoconstriction against which the vasodilator nerves act. Previously we used a prolonged application of a vasoconstrictor agonist to give a sustained constriction, and observed a transient reduction of constriction during and following vasodilator nerve stimulation. Here vasodilator nerve stimulation precedes a brief constrictor stimulus. To help compare the two sets of

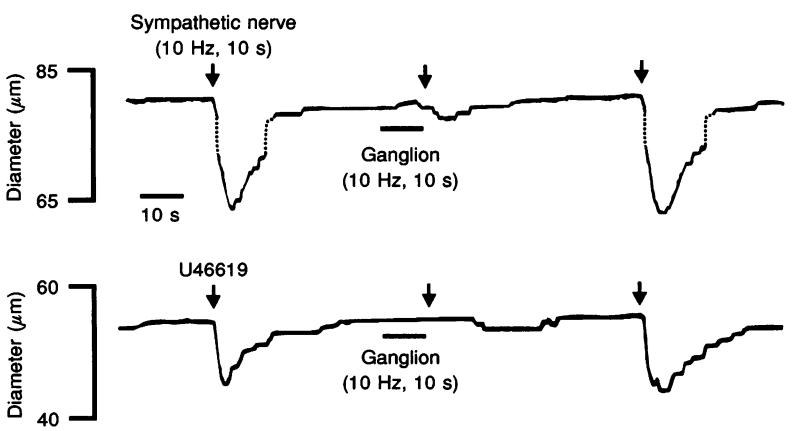

Figure 1. Reduction of vasoconstrictor responses following stimulation of vasodilator nerves in a submucosal ganglion

Small constrictions of submucosal arterioles were evoked either by stimulation of perivascular sympathetic nerves at 10 Hz for 1 s (upper trace) or local application of U46619 from a micropipette (lower trace). Stimulation of a nearby submucosal ganglion at 10 Hz for 10 s immediately prior to the vasoconstrictor stimulus greatly reduced the vasoconstriction. Records from 2 different arterioles. Dotted lines in the upper trace indicate brief periods of incorrect tracking of diameter by the computer (see Methods).

results we performed some experiments using brief applications of U46619, a thromboxane mimetic and one of the vasoconstrictor substances we had used in previous experiments. As shown in Fig. 1, a train of ganglion stimulation prior to an application of U46619 greatly reduced the constrictor response. This indicated that the present protocol employing brief constrictions could demonstrate the effect of vasodilator nerves on the arteriolar smooth muscle that we had described previously. We then considered the possibility of additional effects on neurotransmitter release from perivascular nerves, as described below.

Vasodilator nerve stimulation reduces EJP amplitude

When intracellular recordings of the arteriolar smooth muscle membrane potential were made it could be seen that vasodilator nerve stimulation led to a reduction of the amplitude of the excitatory junction potential (EJP) caused by sympathetic nerve stimulation (Fig. 2). The rise time of EJPs is 110 ms (Hirst, 1977), and our stimulus frequency of 10 Hz therefore gave good summation. After the stimulus train the membrane potential returned to its resting value within 1 s, showing that there were no persistent slow conductance changes (Hirst & Neild, 1978; Edwards & Hirst, 1988). In the submucosal arterioles depolarization due to the summation of EJPs can trigger a regenerative depolarization similar to an action potential but graded in amplitude (Hirst, 1977). This 'active response' leads to smooth muscle contraction. The size of the response and the resulting contraction depended on the rate of depolarization; rapid depolarization triggered larger responses. In the example shown in Fig. 2 the control train of sympathetic stimulation produced a small active response, but after vasodilator nerve stimulation the EJPs were too small to give an active response and the constriction was abolished.

The reduction of EJP amplitude observed in these experiments could have been due to a decrease in the amount of neurotransmitter released by the sympathetic nerves or by a fall in membrane resistance in the smooth muscle. If the membrane resistance had changed this would decrease the membrane time constant, and thus change the rate of decay of the EJP (Hirst & Neild, 1978). There would also be a decrease in amplitude, and the amplitude change expected can be calculated (Edwards *et al.* 1976). A reduction in neurotransmitter release would give a smaller EJP with the same rate of decay as the control.

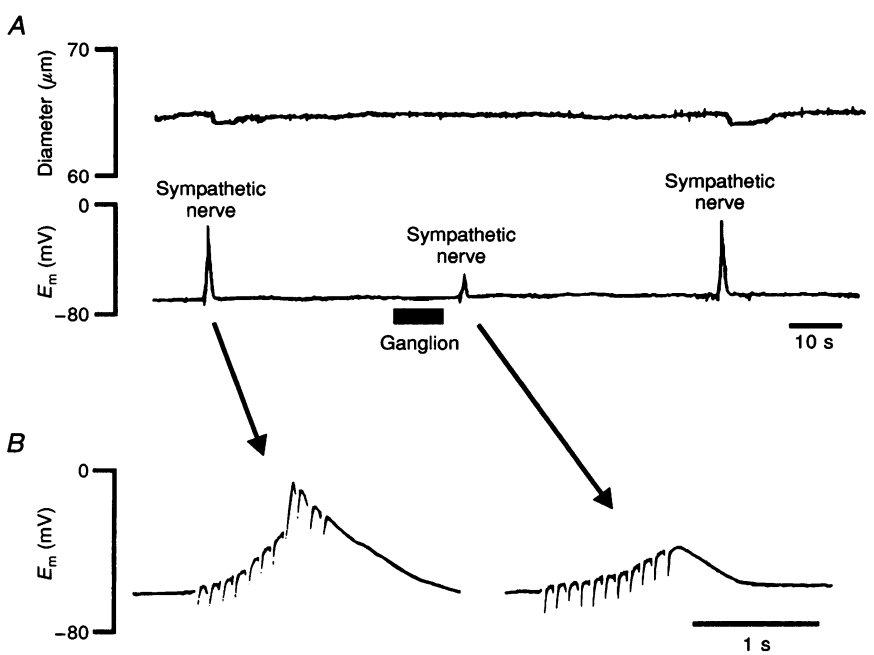

Figure 2. Reduction of EJP amplitude and time constant by a conditioning train of vasodilator nerve stimulation

Simultaneous recordings of arteriolar diameter and smooth muscle membrane potential (*A*). Perivascular nerves were stimulated at 10 Hz for 1 s (Sympathetic nerve) to give EJPs which summed to reach threshold for an active response and caused a small constriction (*B*). Each EJP is preceded by a downward-going stimulus artifact. When perivascular nerve stimulation was preceded by submucosal ganglion stimulation at 10 Hz for 10 s (Ganglion) the size of the EJPs was reduced and there was no active response or constriction. For measurement of the change in EJP amplitude and time constant the effect of ganglion stimulation on single EJPs was observed. These EJPs were too small to evoke an active response and constriction.

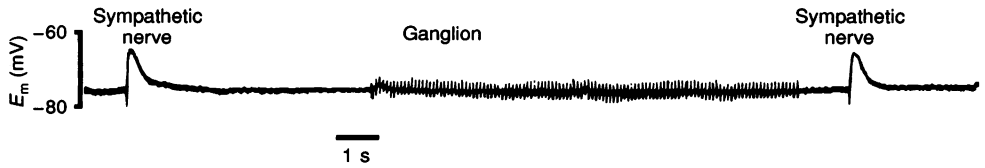

Figure 3. Measurement of the change in EJP amplitude and time constant by observing the effect of ganglion stimulation (10 Hz for 10 s) on single EJPs

EJPs were evoked by a single perivascular nerve stimulus every 17 s. The stimulus voltage was adjusted to give an EJP of approximately 10 mV amplitude, which was too small to evoke an active response and constriction. A submucosal ganglion was stimulated at 10 Hz for 10 s, and an EJP evoked 1 s after the end of the stimulus train. Ganglion stimulation had no effect on the smooth muscle membrane potential, although small stimulus artifacts are visible.

In order to determine whether the shape of the EJP was changed by vasodilator nerve stimulation EJPs were evoked by a single stimulus to the perivascular nerves once every 17 s. The stimulus strength was adjusted to give an EJP of approximately 10 mV amplitude, which was too small to trigger an active response or cause a constriction. When a constant control amplitude of EJP had been established a submucosal ganglion was stimulated at 10 Hz for 10 s, and an EJP evoked 1 s after the end of the stimulus train (Fig. 3). The amplitudes and time constants of the EJPs were measured using a computer system. For control values the three EJPs preceding vasodilator nerve stimulation were averaged to reduce noise in the record; individual EJPs were analysed after the vasodilator stimulation.

Vasodilator nerve stimulation reduced the amplitude of the EJP from $9{\cdot}93 \pm 0{\cdot}40$ to $8{\cdot}90 \pm 0{\cdot}38$ mV ($n = 48$). The reduction was statistically significant ($P < 0{\cdot}001$) as judged by Student's paired t test. The time constant of decay of the EJP was also significantly ($P < 0{\cdot}05$) reduced, from $272 \pm 17{\cdot}0$ to $237 \pm 13{\cdot}9$ ms.

The reduction in EJP decay time constant probably results from a fall in membrane resistance in the smooth muscle. The fall in membrane resistance will also reduce the EJP amplitude, by an amount that can be calculated (Edwards *et al.* 1976). We therefore calculated the amplitude of the EJP expected if the only effect of vasodilator nerve stimulation was to reduce the membrane resistance by the amount indicated by the change in time constant. In all cases the expected amplitude change was very small, and the mean amplitude would have been reduced by only 0·21 mV to $9{\cdot}72 \pm 0{\cdot}41$ mV. As the reduction observed was much greater than this (1·03 mV) we conclude that there must also have been a reduction in the amount of neurotransmitter released from the sympathetic nerve. It is also possible that the same amount of neurotransmitter was released, but that changes had taken place in the smooth muscle cell to reduce the number of channels that opened, or perhaps the average time for which they were open. We assume an effect on neurotransmitter release only because such effects are common and well-documented in a great many neuronal systems.

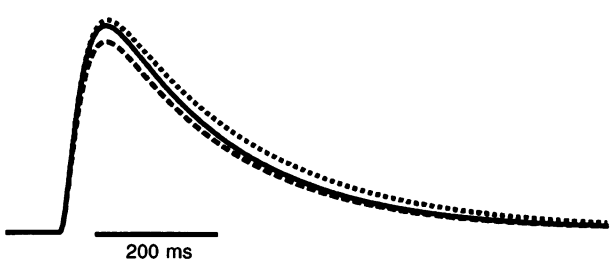

Figure 4. Diagrammatic representation of the effect of vasodilator nerve stimulation on EJP amplitude and time constant of decay

Traces calculated using eqn (2). The control EJP (dotted line) was drawn using the mean amplitude (9·93 mV) and time constant (273 ms) from 48 experiments. The dashed line shows the mean EJP observed after a 10 Hz 10 s train of vasodilator nerve stimulation; it was smaller and decayed more rapidly than the control (amplitude, 8·90 mV; time constant, 237 ms). The continuous line is the calculated EJP assuming that the amount of transmitter released was unchanged after vasodilator nerve stimulation, and only the smooth muscle membrane time constant had changed (by an amount indicated by the fall in time constant of decay). Clearly the calculated EJP (amplitude, 9·72 mV) is not a good fit to the data, suggesting that transmitter release had been decreased.

DISCUSSION

Our previous work has shown that the vasodilator nerves of the submucosal plexus release substances (mainly ACh) that relax the postjunctional arteriolar smooth muscle (Kotecha & Neild, 1995). In those experiments the arteriole was constricted by application of a vasodilator drug; they illustrated the effect of vasodilator nerves on the arteriolar muscle but gave no information about the interaction between vasoconstrictor and vasodilator nerves. The present experiments show that the vasodilator nerves may also substantially reduce the vasoconstriction caused by sympathetic nerve stimulation, and that this may in part be due to a reduction of neurotransmitter from the sympathetic nerves.

The reduction of sympathetic neurotransmitter release by vasodilator nerves is a new finding; although pre-junctional ACh receptors on sympathetic nerves have been demonstrated in many tissues including arteries (Komori & Suzuki, 1987; Fernandes *et al.* 1991), attempts to show that they can be activated by nerve-released ACh have been unsuccessful (Duckles & Kennedy, 1982). The ability of pirenzepine to attenuate the effects of vasodilator nerves supports the role of ACh as the main vasodilator neurotransmitter. Its prejunctional role in our arterioles has also been shown (Kotecha & Neild, 1993); exogenous application of muscarinic agonists attenuates constriction caused by perivascular stimulation, probably by an action on muscarinic M_2 receptors on the sympathetic nerve terminals.

As pirenzepine did not completely abolish the effect of vasodilator nerve stimulation, some of the other potential neurotransmitters which are present in submucosal vasodilator neurones present may also play a role. These include galanin, dynorphin and VIP. Galanin has been shown to reduce the release of VIP (Harling, Gregersen, Rasmussen, Poulsen, Holst & Jenson, 1990; Fox-Threlkeld, McDonald, Cipris, Woskowska & Daniel, 1991) and somatostatin (Madaus, Schusdziarra, Seufferlein & Classen, 1988) from enteric neurones. Dynorphin reduces neurotransmitter release in a number of systems, including some arteries (e.g. Budai & Duckles, 1988). However, some workers report that opioid δ-receptors modulate neurotransmitter release from perivascular sympathetic nerves (Illes, Ramme & Starke, 1986), and dynorphin has a low affinity for them. VIP appears not to modulate transmitter release in any region of the autonomic nervous system.

The structure of the sympathetic nerves around the guinea-pig submucosal arterioles has been examined in detail (Luff, McLachlan & Hirst, 1987; Luff & McLachlan, 1988). No specialized junctions between nerves have been seen that might account for the interaction between vasodilator and sympathetic nerves described here, although close approaches between nerve axons in paravascular nerve bundles is common (Dr S. E. Luff, personal communication; Fig. 1 in Luff *et al.* 1987). The putative vasodilator nerves that run in these bundles are immunoreactive for choline acetyltransferase (Brookes *et al.* 1991; Dr P. A. Steele, personal communication), whereas the VIP-immunoreactive neurones tend to run alone along the surface of the arterioles and are not closely associated with the sympathetic nerves (Galligan *et al.* 1988). It therefore seems unlikely that any of the reduction in sympathetic neurotransmitter release was mediated by VIP.

Our analysis of the EJP revealed a relatively small decrease in amplitude after a train of vasodilator stimuli, whereas data using trains of perivascular stimulation as shown in Fig. 2 suggest a much larger effect. In Fig. 2 the depolarization produced by the summation of the first six EJPs was halved by the vasodilator stimulus. This was due to the failure of the usual facilitation of transmitter release that normally leads the EJP to increase in amplitude during the train (Hirst, 1977). We have not studied this effect further; our analysis of the EJP could not incorporate variations of transmitter release such as facilitation and we were confined to analysing single EJPs. Inspection of records such as Fig. 2 suggests that vasodilator nerves have a much greater effect on transmitter release during trains, and the physiological significance of our results may be greater than the data from single EJPs implies. If the pre- and postjunctional actions of the vasodilator nerves can be selectively blocked (Kotecha & Neild, 1993) it might then become possible to determine which effect plays a greater role in the normal regulation of intestinal blood flow.

We conclude that the intrinsic vasodilator neurones of the intestine exert a powerful effect on the arterioles of the submucosa and can greatly reduce the constrictor effect of the extrinsic sympathetic nerves. Our results are consistent with a dual action of vasoconstrictor nerves, in which they decrease release of vasoconstrictor neurotransmitters from sympathetic nerves as well as acting on the smooth muscle of the arteriole itself.

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