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INTRINSIC AND EXTRINSIC INHIBITORY SYNAPTIC INPUTS TO SUBMUCOUS NEURONES OF THE GUINEA-PIG SMALL INTESTINE

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SUMMARY

1. The sources of inhibitory synaptic inputs to neurones in submucous ganglia of the guinea-pig small intestine were examined by making lesions to cause selective degeneration of nerve terminals of sympathetic or intrinsic origin. Intracellular recordings were used to evaluate the effects of lesions on the inhibitory inputs. Immunohistochemical techniques were used to identify the neurochemical classes of the impaled neurones and to confirm the efficacy of the lesions.

2. The neurones from which recordings were taken were filled with the fluorescent dye Lucifer Yellow. The preparations were then fixed and processed for immunohistochemistry.

3. Thirty-one neurones reactive for vasoactive intestinal polypeptide (VIP) were examined in control submucous ganglia and all exhibited inhibitory synaptic potentials. In preparations extrinsically denervated by severing the mesenteric nerves, twenty-seven of twenty-eight VIP-reactive neurones had inhibitory synaptic potentials. This indicates that these neurones receive inhibitory synaptic inputs from intrinsic neurones. However, significantly more stimuli were required to evoke a detectable inhibitory synaptic potential in extrinsically denervated preparations than in normal intestine.

4. Extrinsic denervations were combined with removal of the myenteric plexus so that nerve terminals arising from both cell bodies in extrinsic ganglia and in the myenteric plexus degenerated. Under these conditions no inhibitory synaptic potentials could be recorded in any of the nine VIP-reactive neurones studied.

5. The conductance change underlying the intrinsic inhibitory synaptic potentials appeared to be similar to that underlying the responses in normal intestine.

6. The time courses of the intrinsic inhibitory synaptic potentials differed from those of the control responses. The responses to short trains of stimuli were significantly briefer and the responses to long trains significantly more prolonged in the extrinsically denervated preparations than in normal preparations.

7. The intrinsic inhibitory synaptic potentials were not significantly affected by phentolamine (0·2 μM), guanethidine (1 μM) or naloxone (1 μM), although the first two drugs markedly depressed control inhibitory synaptic potentials.

8. Extrinsic denervation had no significant effect on the incidence, threshold or

amplitude of the slow excitatory synaptic potentials recorded in VIP-reactive neurones. Slow excitatory synaptic potentials were also observed in some VIP-reactive neurones when extrinsic denervation was combined with removal of the myenteric plexus.

9. It is concluded that VIP-reactive submucous neurones receive inhibitory synaptic input both from extrinsic noradrenergic neurones and from myenteric neurones employing a transmitter that has yet to be identified. These submucous neurones also receive slow excitatory input from neurones intrinsic to the intestine.

INTRODUCTION

Many neurones in the submucous plexus of the guinea-pig small intestine exhibit inhibitory synaptic potentials in response to electrical stimulation of the internodal strands (Hirst & McKirdy, 1975; Surprenant, 1984; Bornstein, Costa & Furness, 1986). Both dopamine and noradrenaline mimic the inhibitory synaptic potentials in those neurones in which such responses can be evoked (Hirst & Silinsky, 1975). The inhibitory synaptic potentials can be blocked by high concentrations of guanethidine (Hirst & McKirdy, 1975), α_2-receptor antagonists (Surprenant, 1984; North & Surprenant, 1985) and 6-hydroxydopamine applied *in vitro* (North & Surprenant, 1985). These results have led to the proposal that the inhibitory responses are mediated by noradrenaline released from sympathetic nerve terminals (North & Surprenant, 1985). Recent findings that inhibitory synaptic potentials are observed almost exclusively in neurones immunoreactive for vasoactive intestinal peptide (VIP) (Bornstein *et al.* 1986) and that these neurones appear to be preferentially innervated by noradrenergic terminals (Furness, Costa & Keast, 1984; Costa & Furness, 1984) support this suggestion.

The results of Hirst & McKirdy (1975), however, appear to be in conflict with the idea that the inhibitory synaptic potentials are mediated by noradrenaline. They found that extrinsic denervation of a segment of small intestine did not alter the incidence of inhibitory synaptic potentials in the submucous neurones in that segment. There are no noradrenergic neurones intrinsic to the guinea-pig small intestine and so any noradrenergic terminals in the submucous plexus must arise from the extrinsic, sympathetic ganglia and degenerate after extrinsic denervation (Costa & Furness, 1971; Furness, Costa & Freeman, 1979; Howe, Provis, Furness, Costa & Chalmers, 1981). Hirst & McKirdy (1975) concluded that the inhibitory synaptic potentials arise from intrinsic neurones.

The aim of this present study was to determine whether inhibitory synaptic potentials arise from terminals of intrinsic neurones, extrinsic neurones or both. The contribution of the extrinsic noradrenergic neurones was assessed by examining the effects of extrinsic denervation on the incidence and properties of the inhibitory synaptic potentials. The source of the intrinsic inhibitory synaptic potentials was examined by combining extrinsic denervation with removal of the myenteric plexus (myectomy). Impaled neurones were filled with the fluorescent dye Lucifer Yellow so that they could be identified after the preparations had been processed for immunohistochemical localization of VIP since the inhibitory synaptic potentials are confined to VIP-reactive neurones (Bornstein *et al.* 1986). The efficacy of the

extrinsic denervations was monitored in the appropriate ganglia by counter-staining the preparations to reveal any tyrosine hydroxylase-like immunoreactivity that persisted in terminals near the impaled neurones. The efficacy of the combined extrinsic denervations and myectomies was determined by counter-staining the preparations to reveal substance P-reactive nerve terminals; these combined lesions cause all the substance P-reactive terminals in the submucosa to degenerate (Costa, Furness, Llewellyn-Smith & Cuello, 1981).

A brief report of some of these results has been published elsewhere (Bornstein, Furness & Costa, 1986).

METHODS

Preparations of submucous plexus from lesioned and control segments of intestine were studied. The methods used for dissection, intracellular recording, focal stimulation of the interganglionic connectives and filling the impaled neurones with the fluorescent dye Lucifer Yellow CH have been described in detail elsewhere (Hirst & McKirdy, 1975; Bornstein, Costa, Furness & Lees, 1984; Bornstein *et al.* 1986; Bornstein, Furness & Costa, 1987). The preparations were bathed in physiological saline (composition in mM: NaCl, 120; KCl, 5·0; $CaCl_2$, 2·5; $MgSO_4$, 1·0; NaH_2PO_4, 1·0; $NaHCO_3$, 25; glucose, 11·5; equilibrated with 95% O_2 and 5% CO_2) which was continuously superfused at 3 ml/min, maintaining the preparation at 34 °C. Drugs were added to the organ bath by changing the reservoir from which the bathing solution was drawn.

Two types of lesioned preparations were used: extrinsic denervations and extrinsic denervation plus myectomies. The lesions were made using methods described previously (Furness & Costa, 1979; Costa & Furness, 1984). Guinea-pigs (180–295 g) were anaesthetized with a mixture of droperidol (10 mg/kg), fentanyl (0·2 mg/kg) and sodium pentobarbitone (15 mg/kg), a segment of small intestine was exteriorized through a mid-line abdominal incision and the perivascular nerves running to that segment were crushed to produce an extrinsic denervation of the segment. In some cases, an 8–12 mm-wide ring of myenteric plexus and longitudinal muscle was also removed (myectomy) from the segment of intestine that had been extrinsically denervated. The abdomen was then closed and the animal allowed to recover. The operated animals were killed after 7–10 days when the nerve terminals isolated from their cell bodies by the lesions had degenerated.

The efficacy of the lesions was assessed immunohistochemically in each ganglion in which a neurone had been impaled. Impaled neurones were readily identified as they were filled with Lucifer Yellow which remains fluorescent after fixation and processing for immunohistochemical localization of peptides (Bornstein *et al.* 1984). The procedure followed to identify the peptide content of an impaled neurone has been described in detail elsewhere (Bornstein *et al.* 1986, 1987). After each experiment the preparation was fixed and stained for VIP-reactivity using a primary antibody raised in a rabbit (code 7913 from Dr J. H. Walsh) and a secondary anti-rabbit antibody coupled to tetramethylrhodamine isothiocyanate (Cappel Laboratories or Silenius); this allowed the VIP-reactivity of the impaled neurones to be assessed. If one or more of the impaled neurones was found not to be immunoreactive for VIP then the preparation was restained to reveal neuropeptide Y (NPY)-like immunoreactivity with a primary antibody raised in a rabbit (code JBM 263/1 from Drs C. Maccarrone and B. Jarrott) and the same rhodamine-coupled secondary antibodies. Neurones that were not reactive were classified as VIP,NPY-negative. The presence of extrinsic terminals was assessed simultaneously with the first staining procedure by staining the preparation to reveal tyrosine hydroxylase (TH) immunoreactivity using a monoclonal primary antibody raised against phenylalanine hydroxylase that is known to cross-react with TH (Macrae, Furness & Costa, 1986). In this case, the secondary antibody was an antimouse antibody coupled to fluorescein isothiocyanate (FITC). The Lucifer Yellow fluorescence and that of the FITC were distinguished using Leitz D and L3 filter systems. The D filter system allows visualization of Lucifer Yellow alone, whereas with the L3 filter system both Lucifer Yellow and FITC, but not rhodamine, are seen. Rhodamine was visualized with a Leitz N2 filter system with which Lucifer Yellow and FITC are not seen. This meant that by using appropriate filter combinations it was possible to distinguish between the Lucifer Yellow fluorescence of the impaled neurones, any VIP-

reactivity of these cells and the TH-reactivity of extrinsic terminals. Neurones in ganglia with any TH-reactive varicosities were not considered to be adequately denervated. In each case, a piece of unoperated submucosa was processed in parallel with the experimental preparation to verify that the histochemical procedures had been adequate. When extrinsic denervation plus myectomies were performed the efficacy of the lesions was monitored in a similar fashion but a monoclonal anti-substance P antibody (Cuello, Galfré & Milstein, 1979) was used in place of the anti-TH antibody. This was because substance P-reactive terminals in the submucosa come from both sensory and myenteric ganglia (Costa *et al.* 1981) so that all such terminals disappear only when both an extrinsic denervation and a myectomy have been carried out successfully. If no substance P-reactive terminals were present, the preparations were restained for TH to provide further confirmation.

Measurements of the amplitudes of inhibitory synaptic potentials and slow excitatory synaptic potentials were made from the resting membrane potential prior to the period of stimulation to the peak of the response. The duration of each inhibitory synaptic potential was taken to be the time from the beginning of the hyperpolarization until the membrane potential returned to its initial value.

RESULTS

The synaptic inputs to eighty-one neurones in thirty-one control preparations, fifty-five neurones in twenty-three extrinsically denervated preparations and twenty-four neurones in seven preparations that had undergone both extrinsic denervation and myectomy operations were characterized electrophysiologically. As has been reported previously (Bornstein *et al.* 1986), five different types of synaptic potential could be recorded in control neurones. These were fast excitatory synaptic potentials, inhibitory synaptic potentials, slow excitatory synaptic potentials, intermediate excitatory synaptic potentials and multipulse slow excitatory synaptic potentials. The latter two types of synaptic potential are observed relatively infrequently (Bornstein *et al.* 1986) and so have not been included in the analysis described below.

Immunohistochemical analysis showed that thirty-one of the control neurones were immunoreactive for VIP, fourteen were immunoreactive for NPY, and fifteen were not immunoreactive for either of these peptides. Of the remainder, seven could not be found after processing for immunohistochemistry, seven were in preparations where the VIP-like immunoreactivity was too faint to allow the neurones to be classified and seven were impaled with electrodes containing 1 M-KCl and no Lucifer Yellow.

In the preparations that had been extrinsically denervated, thirty-three neurones were found to be VIP-reactive, but TH-reactive varicosities were observed near five of these cells which were excluded from further analysis. Seven of the denervated neurones were NPY-reactive, eleven were VIP,NPY-negative and four were in preparations whose VIP-immunoreactivity was too weak to allow the neurones to be classified although the absence of TH-reactive terminals was confirmed.

Twelve VIP-reactive neurones were characterized in the preparations that had undergone both extrinsic denervation and myectomy, but three of these were in ganglia in which either substance P- or TH-reactive varicosities could still be detected. Two NPY-reactive neurones were characterized in these preparations, five neurones were VIP,NPY-negative and five could not be identified immunohistochemically.

Effects of the lesions on the incidence of synaptic potentials

It has previously been reported that inhibitory synaptic potentials are found almost exclusively in VIP-reactive submucous neurones and that almost all such neurones exhibit such responses (Bornstein *et al.* 1986, 1987). The results obtained in this present study confirm these observations (Table 1) as all thirty-one control VIP-reactive neurones exhibited inhibitory synaptic potentials and these responses were not seen in any of the other neurochemically identified neurones. Extrinsic denervation did not appear to alter this distribution of responses (Table 1).

TABLE 1. Incidence of fast excitatory synaptic potentials (fast ESPs), inhibitory synaptic potentials (ISPs) and slow excitatory synaptic potentials (slow ESPs) in immunohistochemically identified neurones in control and lesioned preparations

Lesion	Cell type	*n*	Fast ESPs	ISPs	Slow ESPs
None	VIP-reactive	31	31	31	31
	NPY-reactive	14	14	0	0
	VIP, NPY-negative	15	15	0	0
Extrinsic denervation	VIP-reactive	28	28	27	28
	NPY-reactive	7	7	0	0
	VIP, NPY-negative	11	11	0	1
Myectomy*	VIP-reactive	19	18	17	6
	NPY-reactive	7	7	0	0
	VIP, NPY-negative	7	5	0	0
Extrinsic denervation plus myectomy	VIP-reactive	9	9	0	5
	NPY-reactive	2	2	0	0
	VIP, NPY-negative	5	5	0	0

* Results from myectomy preparations were taken from Fig. 2 and Table 1 of Bornstein *et al.* (1987).

Inhibitory synaptic potentials could be evoked in twenty-seven of the twenty-eight VIP-reactive neurones in the ganglia for which extrinsic denervation was confirmed histochemically (e.g. Fig. 1). The lesions did not alter the incidence of slow excitatory synaptic potentials in the VIP-reactive neurones (Fig. 1 and Table 1) nor did they induce responses that could not be observed in control cells in the other neurochemical cell types (Table 1). Thus, both inhibitory synaptic potentials and slow excitatory synaptic potentials can be mediated by nerve terminals derived from cell bodies intrinsic to the intestine.

When extrinsic denervation was combined with removal of the myenteric plexus, no inhibitory synaptic potentials could be evoked in any of the nine VIP-reactive neurones for which the efficacy of the lesions could be confirmed (Table 1; e.g. Fig. 2). Thus, the intrinsic nerve terminals responsible for the inhibitory synaptic potentials probably have their cell bodies in the myenteric plexus. In contrast, although the incidence of the slow excitatory synaptic potentials was significantly reduced ($P < 0{\cdot}01$, χ^2 test, 1 degree of freedom), such responses were observed in five of these neurones (Fig. 3) and all exhibited fast excitatory synaptic potentials.

As these results indicate that extrinsic denervation does not significantly alter the incidence or distribution of the inhibitory synaptic potentials, and as these responses

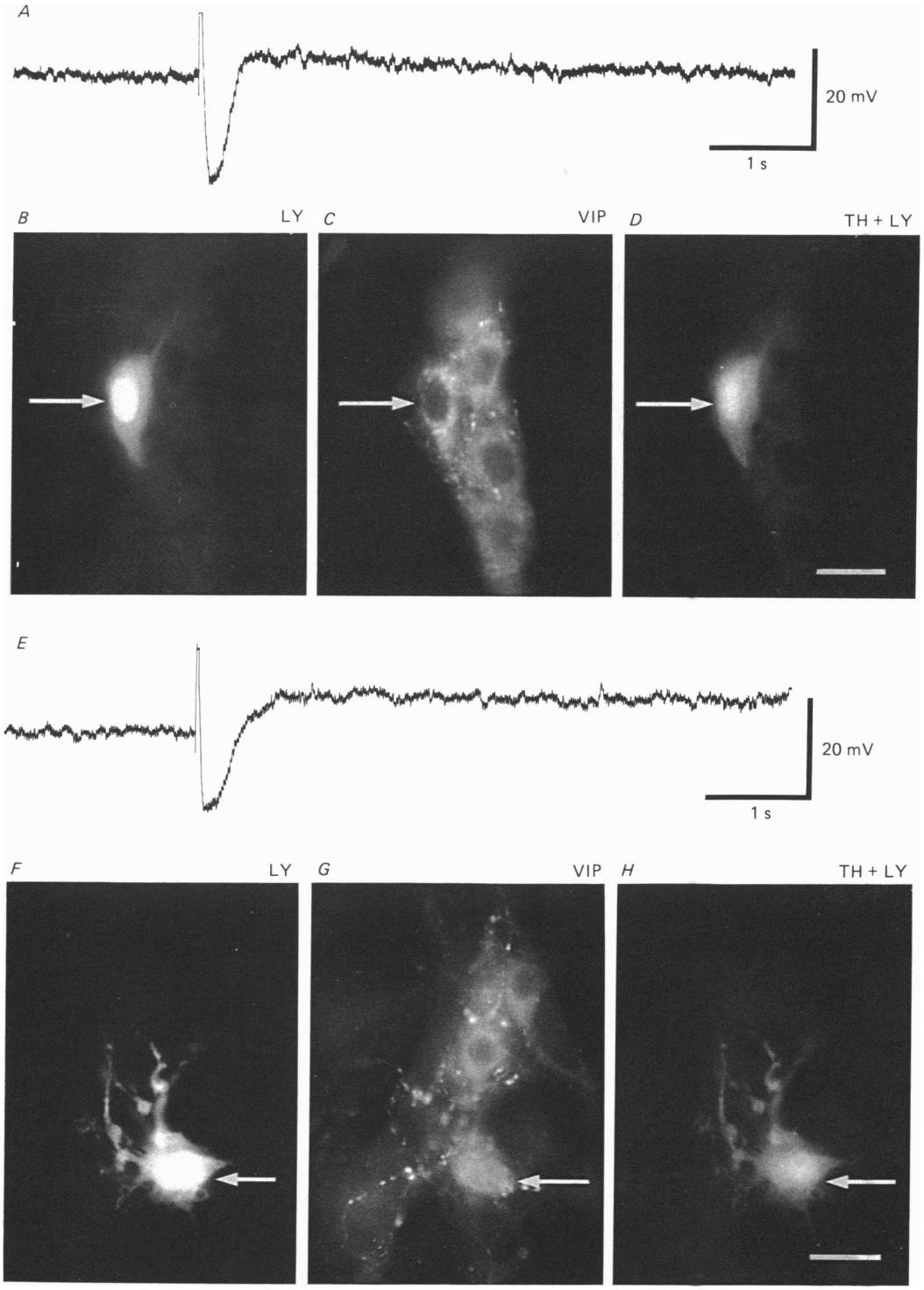

Fig. 1. For legend see opposite.

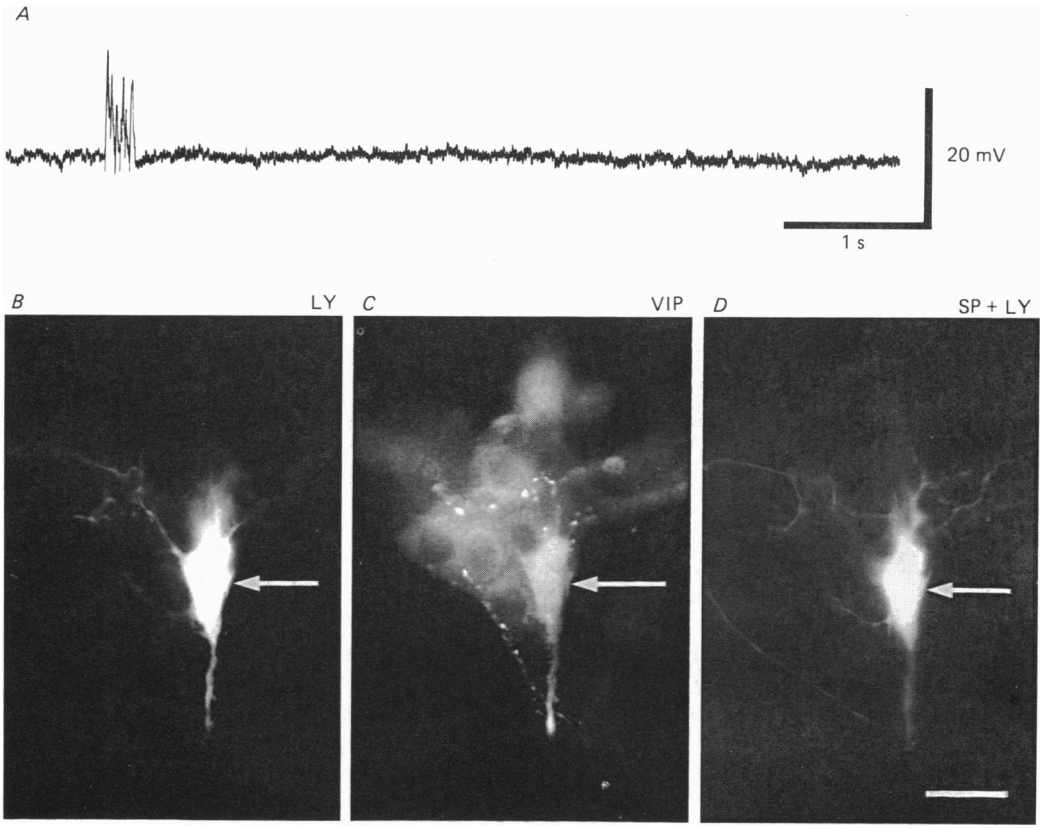

Fig. 2. An example of the absence of both inhibitory synaptic potentials and slow excitatory synaptic potentials from VIP-reactive neurones in a preparation that had undergone both extrinsic denervation and myectomy. *A* shows the response to focal stimulation of an interganglionic connective with a train of 5 pulses at 30 Hz; only fast excitatory synaptic potentials were evoked. *B* shows the Lucifer Yellow-filled neurone from which the recording was taken. *C* shows the VIP-reactivity of the ganglion containing the neurone (arrow). *D* shows the absence of substance P-reactive structures in the ganglion indicating that the lesions were successful (the Lucifer Yellow fluorescence can also be seen with this filter). Bar = 20 μm.

Fig. 1. Examples of the inhibitory synaptic potentials evoked by focal stimulation in two VIP-reactive neurones in denervated preparations. The inhibitory responses (and the slow excitatory synaptic potentials that followed them) that were recorded with intracellular electrodes are shown in *A* and *E*. The neurones from which the recordings were taken were filled with Lucifer Yellow from the recording electrode and fixed for immunohistochemistry. The Lucifer Yellow fluorescence of these two neurones is shown in *B* and F (these micrographs were taken with a filter that showed only the Lucifer Yellow; see Methods). The VIP-like immunoreactivity of the same microscope fields and hence in each of the two ganglia is shown in *C* and *G* (the arrows show the Lucifer Yellow-filled neurones). *D* and *H* show the same microscope fields photographed with a filter that showed both tyrosine hydroxylase-like immunoreactivity and Lucifer Yellow fluorescence (TH + LY), these micrographs confirm the absence of TH-like immunoreactivity in each ganglion as the only fluorescent structures in the ganglia are the two Lucifer Yellow-filled neurones. The action potentials and fast excitatory synaptic potentials evoked by the stimuli were attenuated by the frequency response of the chart recorder. Bar = 20 μm.

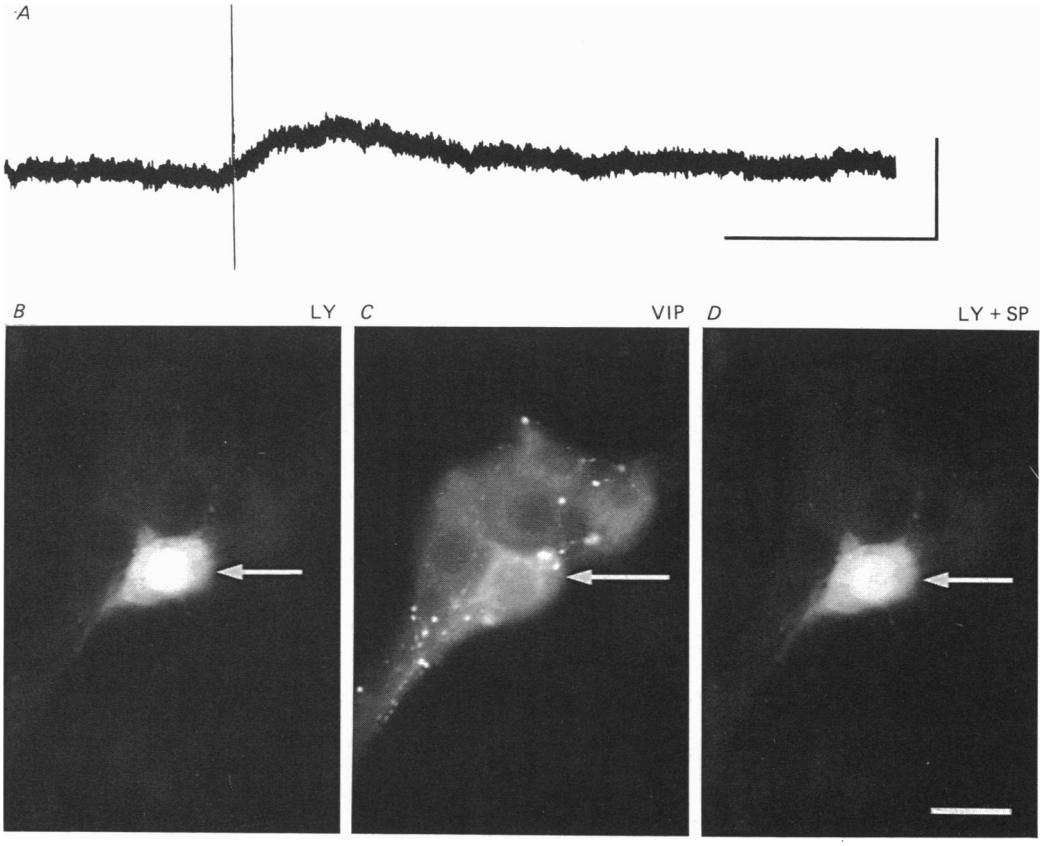

Fig. 3. An example of the presence of a slow excitatory synaptic potential in a VIP-reactive neurone in a preparation that had undergone both extrinsic denervation and myectomy. *A* shows the response to a train of 3 stimuli at 30 Hz; no inhibitory synaptic potential was evoked but there was a slow excitatory synaptic potential. *B* shows the Lucifer Yellow-filled neurone. *C* shows the VIP-reactivity of the ganglion containing this neurone (arrow). *D* shows the absence of substance P-reactive terminals in the ganglion, confirming that the lesions had been effective. Bar = 20 μm.

are confined to VIP-reactive neurones, the analysis of the inhibitory synaptic potentials below include neurones whose VIP-reactivity was not confirmed.

Effects of denervation on the properties of the inhibitory synaptic potentials

The number of pulses in the train of stimuli at 30 Hz required to evoke a detectable inhibitory synaptic potential in control neurones ranged from 1 to 4 with a median of 1·5. In contrast, significantly more pulses were required in the extrinsically denervated preparations where the threshold number of pulses ranged from 1 to 10 with a median of 2·9 (Fig. 4). This difference was significant ($P < 0{\cdot}005$, Wilcoxon rank-sum test (Snedecor & Cochran, 1967)) which suggests that some inhibitory nerve terminals had degenerated as a result of the lesion.

Extrinsic denervation produced marked changes in the time courses of the

inhibitory synaptic potentials. In most control neurones, the inhibitory responses appeared to be monophasic regardless of the number of pulses in the stimulus train (e.g. Fig. 5*A* and *B*). In neurones after extrinsic denervation, the inhibitory synaptic potentials evoked by 10 pulses at 30 Hz were usually biphasic (e.g. Fig. 5*C* and *D*), and the second phase was often very long (up to 55 s). When the durations of

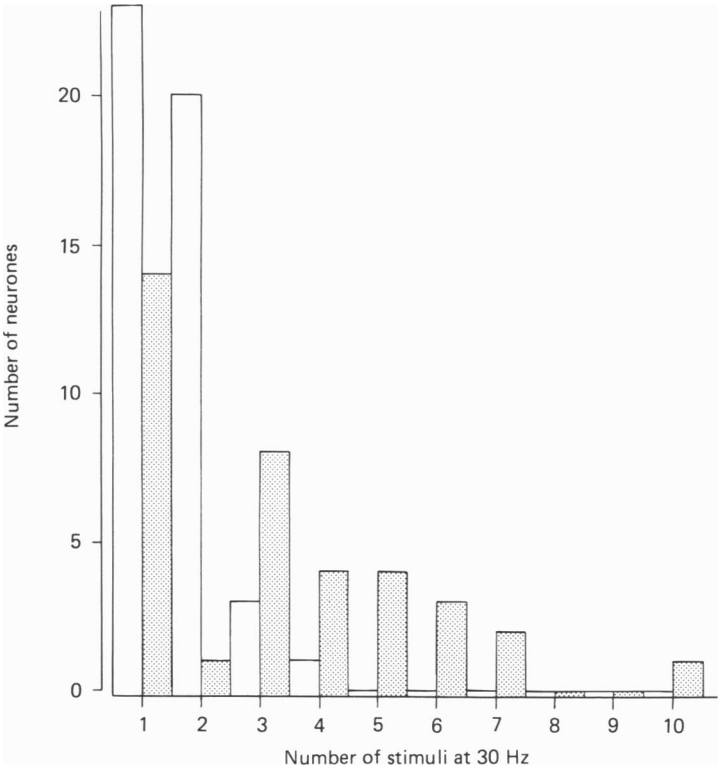

Fig. 4. A histogram showing the effects of extrinsic denervation on the minimum number of stimuli required to evoke inhibitory synaptic potentials in individual neurones. The open bars show the distribution of the number of stimuli (in trains of pulses at 30 Hz) required to evoke a detectable inhibitory synaptic potential in forty-six neurones in control preparations. The stippled bars show the distribution for thirty-seven neurones in extrinsically denervated preparations.

responses to such 10 pulse stimuli in control neurones were compared to those observed in denervated neurones it was found that the responses in denervated cells were significantly longer ($P < 0{\cdot}05$). This difference is even more striking when it is considered that fewer stimuli are required to evoke an inhibitory synaptic potential in the control neurones. In contrast, the responses to short trains of stimuli (1–3 pulses) were much briefer in the denervated preparations than in the controls (e.g. Fig. 11*A* and *C*). Only those neurones in which a single pulse could evoke an inhibitory synaptic potential were compared quantitatively. The durations of the inhibitory synaptic potentials evoked in fifteen control neurones by 3 stimuli at 30 Hz ranged from 660 to 3020 ms with a mean of 1330 ms; in ten denervated

neurones these durations ranged from 220 to 940 ms with a mean of 460 ms ($P < 0{\cdot}01$). Thus, it appears that extrinsic denervation revealed two separate components of the inhibitory synaptic potentials that were not usually recognized in controls.

As might have been expected from the change in the threshold number of pulses required to evoke inhibitory synaptic potentials in denervated preparations, the

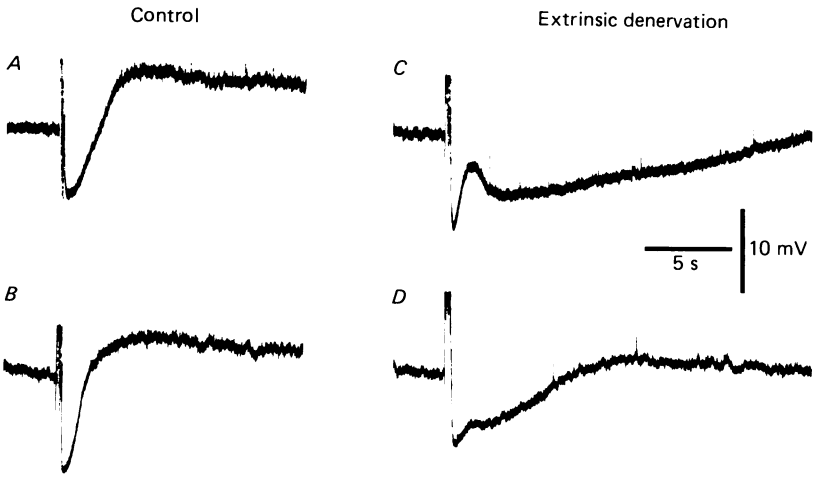

Fig. 5. Comparison of the effects of trains of 10 stimuli at 30 Hz in two neurones from control preparations (*A* and *B*) with the effects of identical trains of stimuli in two neurones in extrinsically denervated preparations (*C* and *D*): in the control neurones the trains evoked monophasic inhibitory synaptic potentials followed by slow excitatory synaptic potentials; in the lesioned preparations the trains evoked biphasic inhibitory synaptic potentials, with any slow excitatory synaptic potentials being masked by the slow phase of the inhibitory response.

mean amplitudes of the responses evoked in such preparations were less than those in controls (Fig. 6*A*). Individual neurones in denervated preparations, however, often exhibited pulse–amplitude curves that were within the control range (Fig. 6*B*).

The briefer component of the inhibitory responses evoked in denervated preparations reversed at about 30 mV negative to the resting membrane potential, as did the control responses (Fig. 7). The slow component reversed at a similar membrane potential (Fig. 8) but as it was usually contaminated by the presence of an underlying slow excitatory synaptic potential an exact determination of the reversal potential was not possible. All three types of inhibitory synaptic potential were associated with marked decreases in the input resistances of the neurones in which they were recorded (Fig. 9). These results suggest that the conductance changes mediating the inhibitory synaptic potentials in denervated preparations may be similar to those mediating control responses.

Effects of extrinsic denervation on the slow excitatory synaptic potentials

The extrinsic denervations had no effect on the number of pulses required to evoke slow excitatory synaptic potentials in VIP-reactive neurones (range 1–2 pulses,

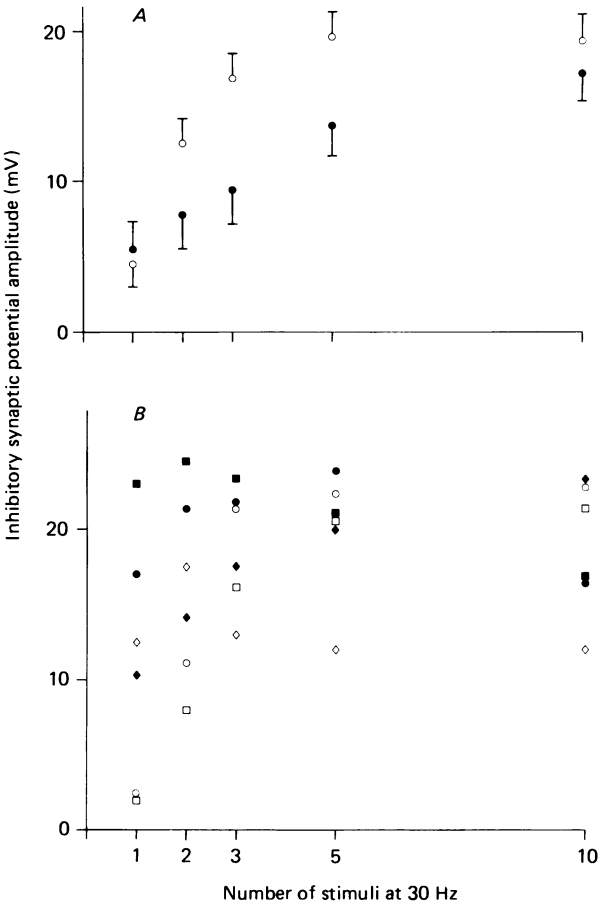

Fig. 6. Comparison of the effects of increasing the number of pulses in a train of stimuli on the amplitudes of the inhibitory synaptic potentials recorded in control and extrinsically denervated preparations. *A* shows the mean amplitudes of the inhibitory synaptic potentials evoked in control (○) and lesioned (●) preparations by 1, 2, 3, 5 and 10 stimuli at 30 Hz (each point represents the mean amplitude of responses from at least thirteen neurones). *B* shows the effects of increasing the number of stimuli in a train on the amplitudes of the inhibitory synaptic potentials in three neurones in control preparations (open symbols) and three neurones in lesioned preparations (filled symbols). These results were chosen to illustrate the variability between neurones and the similarity of some neurones in lesioned and intact preparations.

mean 1·03 for control neurones and 1–2 pulses, mean 1·04 for denervated neurones). Similarly, the amplitudes of the slow excitatory synaptic potentials evoked by single pulses were not significantly altered by these lesions ($P > 0{\cdot}1$). Thus, as there was also no change in the incidence of slow excitatory synaptic potentials after the extrinsic denervations (Table 1), it is unlikely that extrinsic nerves contribute to these responses.

Pharmacological analysis of the intrinsic inhibitory synaptic potentials

It has been reported that the inhibitory synaptic potentials in control cells are blocked by α_2-receptor antagonists (Surprenant, 1984; North & Surprenant, 1985). In the present study, it was found that phentolamine, an α-receptor antagonist, substantially reduced amplitudes and durations of the inhibitory synaptic potentials recorded in control neurones (Fig. 10). The mean duration of the inhibitory synaptic

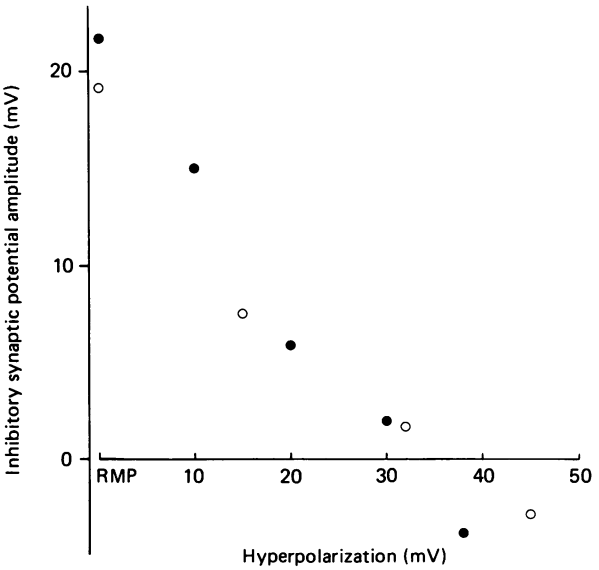

Fig. 7. The effects of varying the membrane potential on the amplitudes of the inhibitory synaptic potentials evoked by 3 stimuli at 30 Hz in a control and a denervated preparation. ○, effects of hyperpolarization on the responses in a control neurone; ●, effects in a neurone in a denervated preparation (each point represents the mean of at least three responses). RMP, resting membrane potential.

potentials evoked by a train of 3 pulses at 30 Hz was reduced from 1230 to 300 ms (six neurones, $P < 0{\cdot}01$) and the mean amplitude fell from 22·8 to 7·3 mV ($P < 0{\cdot}01$). This effect was observed with 0·2 μM-phentolamine and was not consistently altered by increasing the concentration to 2 μM.

Phentolamine, however, had no effect on either component of the inhibitory synaptic potentials in five neurones in extrinsically denervated preparations (Fig. 11); all of these cells exhibited inhibitory synaptic potentials after a single stimulus. The mean amplitudes of the responses evoked by trains of 3 pulses at 30 Hz in drug-free solutions and in 0·2 μM-phentolamine were 12·2 and 12·0 mV, respectively, and the mean durations were 360 and 340 ms, respectively. Thus, it is unlikely that the inhibitory synaptic potentials in these preparations are mediated by α-receptors.

Guanethidine, which depresses the release of noradrenaline, has also been reported to block the inhibitory synaptic potentials in control preparations (Hirst & McKirdy, 1975). In the present study, it was found that although 1 μM-guanethidine

substantially depressed the inhibitory synaptic potentials in four control neurones (Fig. 12*A*), it had little or no effect on similar responses in four neurones in extrinsically denervated ganglia (Fig. 12*B*).

Enkephalin-immunoreactive varicosities are found in submucous ganglia and disappear after the overlying myenteric plexus is removed (Furness, Costa & Miller,

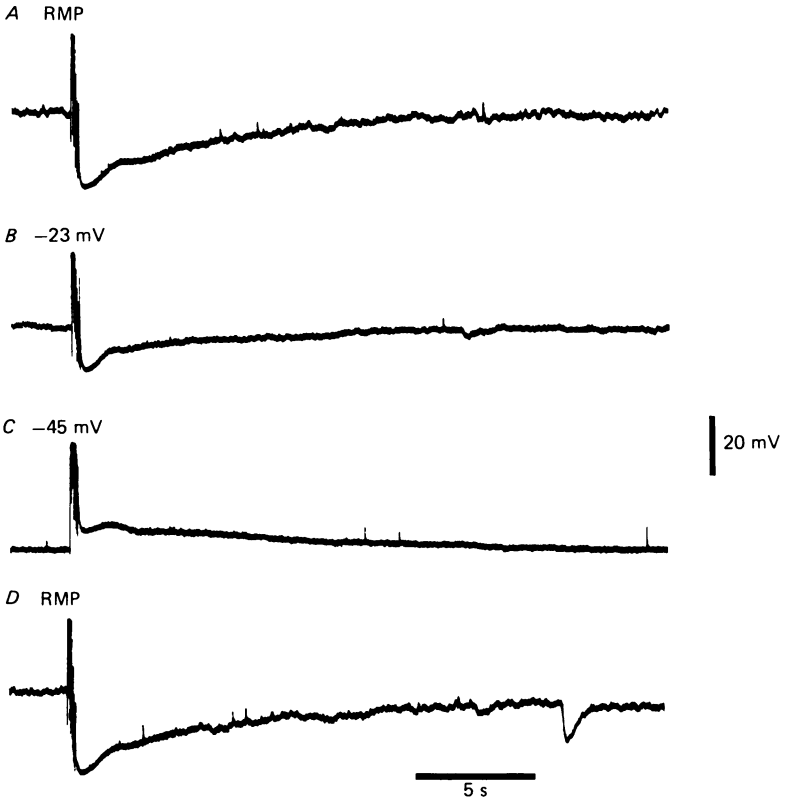

Fig. 8. Effects of maintained hyperpolarization on the slow component of the inhibitory synaptic potential evoked in a neurone in an extrinsically denervated preparation. *A*, the response to a stimulus of 10 pulses at 30 Hz starting from the resting membrane potential (RMP). *B*, the response to a similar stimulus when the membrane had been hyperpolarized by 23 mV. *C*, the response seen when the membrane had been hyperpolarized by a total of 45 mV. *D*, the response seen when the membrane potential had been returned to its normal resting level. An apparent spontaneous inhibitory synaptic potential can be seen in the trace shown in *D*.

1983). Furthermore, enkephalin hyperpolarizes submucous neurones of both the small intestine (Surprenant & North, 1985) and the caecum (Mihara & North, 1986) by an action on δ-receptors. In the present experiments, however, naloxone (1 μM) failed to block either component of the inhibitory synaptic potentials in four neurones in denervated preparations (e.g. Fig. 13).

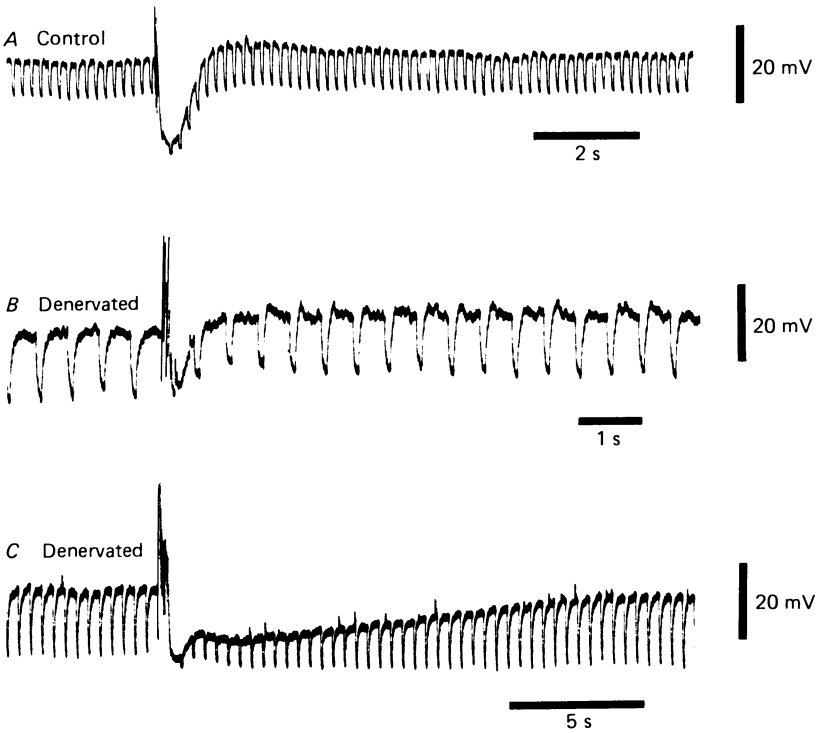

Fig. 9. Changes in the input resistance produced by the inhibitory synaptic potentials evoked by: *A*, 3 stimuli at 30 Hz in a control neurone; *B*, a similar stimulus in an extrinsically denervated neurone; *C*, 10 stimuli at 30 Hz in another extrinsically denervated neurone. In each case the inhibitory synaptic potential is accompanied by reductions in the amplitudes of the hyperpolarizing responses to intracellular current injections, i.e. by decreased membrane resistance.

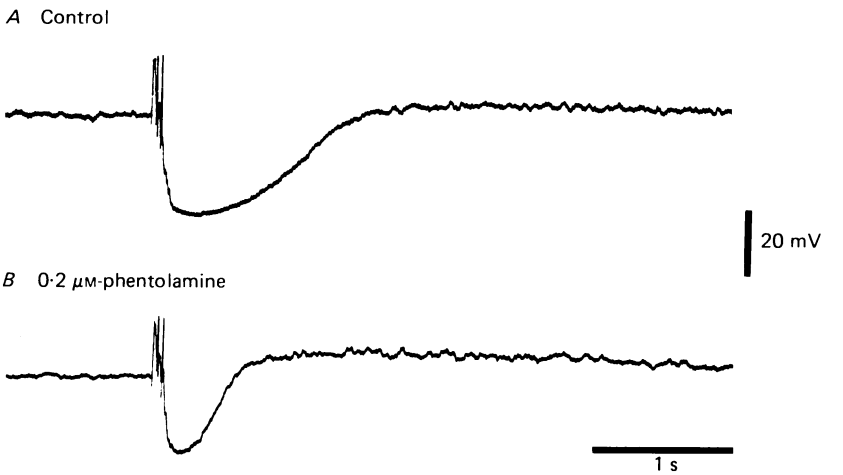

Fig. 10. The effect of 0·2 μM-phentolamine on the response of a control neurone to 3 stimuli at 30 Hz. The inhibitory synaptic potential in phentolamine (*B*) is considerably briefer and smaller than that seen in control (*A*).

DISCUSSION

These results indicate that the inhibitory synaptic potentials recorded in VIP-reactive submucous neurones result from activation of at least two different types of inhibitory neurones. These are sympathetic noradrenergic neurones whose cell bodies are in the coeliac ganglion and one or more populations of neurones with cell bodies in the myenteric plexus.

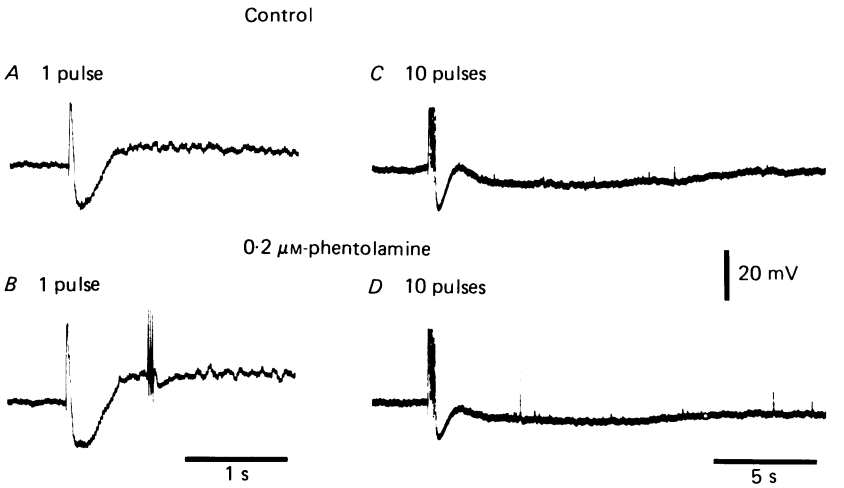

Fig. 11. Examples of the absence of effect of 0·2 μM-phentolamine on the inhibitory synaptic potentials evoked by a single stimulus (*A*, control and *B*, phentolamine) and 10 stimuli at 30 Hz (*C*, control and *D*, phentolamine) in a neurone in an extrinsically denervated preparation.

The evidence that the extrinsic noradrenergic neurones contribute to the inhibitory synaptic potentials is derived from several different types of experiment. The inhibitory synaptic potentials in control preparations can be substantially reduced by α-receptor antagonists (Fig. 9; see also Surprenant, 1984; North & Surprenant, 1985) and guanethidine (Fig. 11; see also Hirst & McKirdy, 1975). In contrast, the inhibitory synaptic potentials recorded after extrinsic denervation were insensitive to concentrations of these drugs that had profound effects on control responses. This suggests that a different transmitter was responsible for the inhibitory synaptic potentials seen after degeneration of the noradrenergic terminals. Furthermore, extrinsic denervation increased the number of stimulus pulses required to evoke an inhibitory synaptic potential and shortened the initial component of the response while revealing a longer late component (Figs 5, 8, 10, 11 and 12). Thus, elimination of the noradrenergic terminals causes marked changes in the properties of the inhibitory synaptic potentials.

Extrinsic denervation did not, however, reduce the proportion of VIP-reactive neurones in which inhibitory synaptic potentials can be evoked. Thus, some of the inhibitory neurones innervating the submucous plexus must be intrinsic to the gut. Conversely, removal of the myenteric plexus without interruption of the extrinsic

nerves does not alter the incidence of inhibitory synaptic potentials in VIP-reactive neurones (Bornstein *et al.* 1987). The inhibitory responses disappear, however, when extrinsic denervation is combined with removal of the myenteric plexus. This indicates that almost all VIP-reactive neurones receive inhibitory input from both extrinsic and intrinsic (myenteric) neurones.

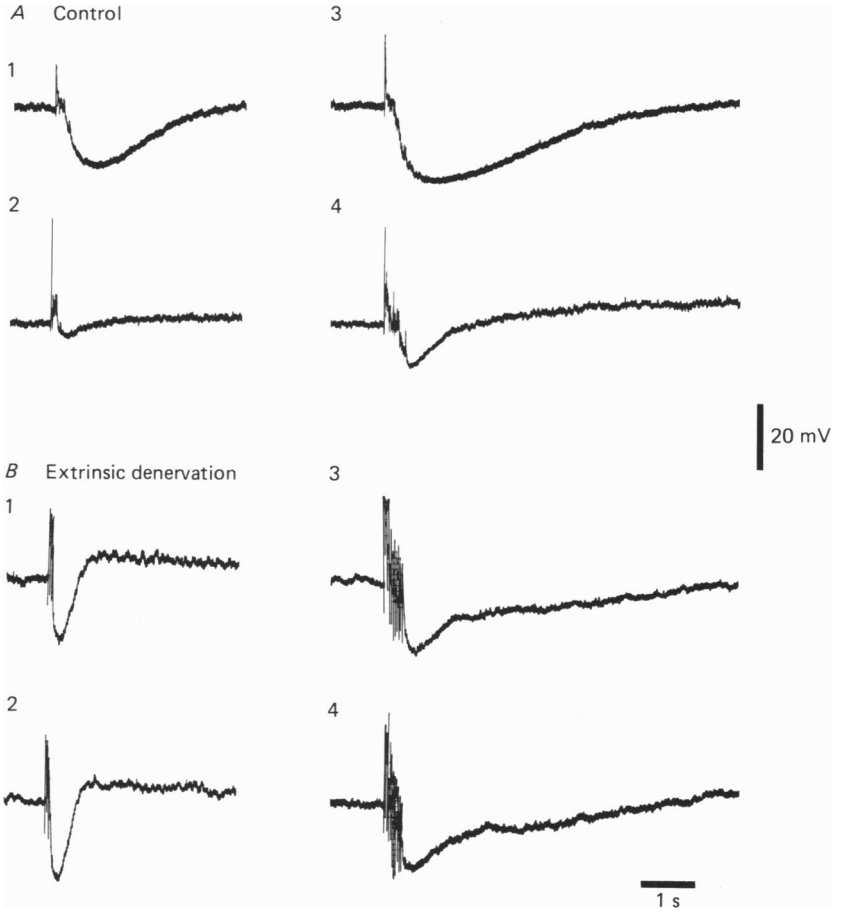

Fig. 12. Effects of 1 μM-guanethidine on the responses to 3 (A 1 and 3; B 1 and 3) and 10 (A 2 and 4; B 2 and 4) stimuli at 30 Hz in a control neurone (A) and a neurone in an extrinsically denervated preparation (B). A 1, A 3, B 1 and B 3 show the responses recorded in control solution and A 2, A 4, B 2 and B 4 show the responses in guanethidine. There was a substantial reduction in the responses recorded in the unoperated preparation but no effect on the responses recorded in the lesioned preparation.

The relative contributions of the intrinsic and extrinsic inhibitory inputs to the inhibitory synaptic potentials recorded in control solutions are not calculable. The effect of phentolamine, however, in shortening but not abolishing the inhibitory synaptic potentials in control neurones, suggests that the intrinsic, non-noradrenergic terminals can make a significant contribution. The early phase of the inhibitory synaptic potentials seen after extrinsic denervation is similar to that seen

in phentolamine-containing solution, which suggests that the two treatments are comparably effective in eliminating transmission from the extrinsic neurones. This, in turn, suggests that there is no significant reorganization of the intrinsic inputs after the lesions.

The transmitter, or transmitters, responsible for the intrinsic inhibitory synaptic potentials is not known. Immunohistochemical studies have revealed the presence of

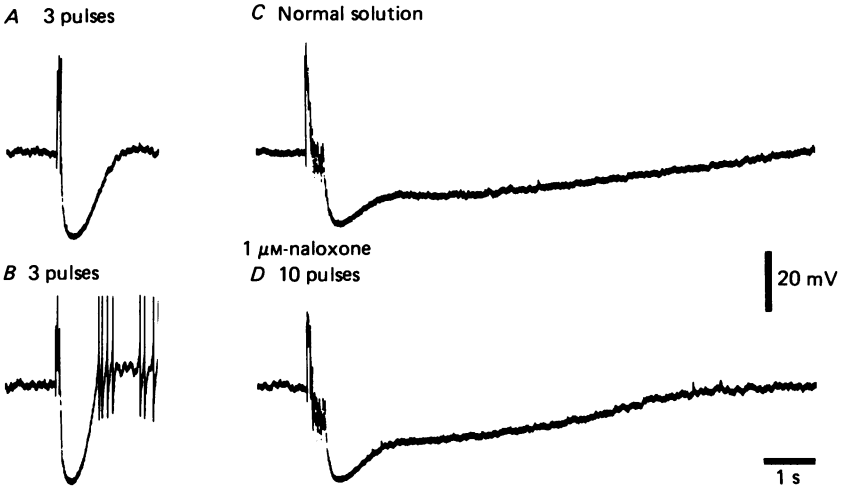

Fig. 13. An example of the inhibitory synaptic potentials recorded in a neurone in an extrinsically denervated preparation before (*A* and *C*) and after 1 μM-naloxone (*B* and *D*). The responses to 3 (*A* and *B*) and 10 (*C* and *D*) stimuli at 30 Hz are shown.

several different peptides in nerve terminals in the submucous ganglia. Two of these peptides, somatostatin and enkephalin, hyperpolarize neurones in the submucous plexus of the guinea-pig caecum via ion channels similar to those opened by noradrenaline (Mihara & North, 1986). Similarly, enkephalin hyperpolarizes submucous neurones from the small intestine (Surprenant & North, 1985). It seems unlikely, however, that the intrinsic inhibitory synaptic potentials are due to an action of enkephalin as 1 μM-naloxone had little or no effect on these responses. There are at least two populations of somatostatin-reactive terminals in the submucous plexus of the small intestine; this peptide is contained in the extrinsic noradrenergic terminals and also in terminals of myenteric origin (Costa & Furness, 1984). An inhibitory role for somatostatin would be consistent with the results of Keast, Furness & Costa (1986) who found that somatostatin, like noradrenaline, depressed the activity of non-cholinergic secretomotor neurones in the submucous plexus. These neurones are thought to be the VIP-reactive neurones in which the intrinsic inhibitory synaptic potentials can be evoked (Bornstein *et al.* 1986; Keast, 1987).

The biphasic nature of many of the inhibitory synaptic potentials of intrinsic origin evoked by bursts of 10 or more stimuli suggest that there may be two different intrinsic inhibitory neurones, or that one inhibitory neurone releases more than one transmitter, or that a single transmitter acts on two different receptors. The

similarity of reversal potentials and of the underlying changes in input resistance between the intrinsic and extrinsic inhibitory synaptic potentials suggest that the intrinsic responses, like the extrinsic responses (North & Surprenant, 1985), are due to an increase in the potassium conductance of the membrane. Both enkephalin and somatostatin open the same ion channels as noradrenaline in the submucous neurones of guinea-pig caecum (Mihara & North, 1986).

Extrinsic denervation had no apparent effect on slow excitatory synaptic potentials that were observed in VIP-reactive neurones; thus the extrinsic nerves do not provide a significant component of this input. The slow excitatory synaptic potentials could be due to one or more of several different peptides, known to excite submucous neurones, that have been identified immunohistochemically in nerve terminals within submucous ganglia. These include substance P and VIP (Costa *et al.* 1981; Costa & Furness, 1983; Surprenant, 1984; Mihara, Katayama & Nishi, 1985). The substance P terminals disappear from the submucosa after the overlying myenteric plexus is removed from an extrinsically denervated segment of intestine (Costa *et al.* 1981; see also Figs 2 and 3). Thus, if substance P mediates the slow excitatory synaptic potentials then these responses would be expected to disappear from such denervated preparations. However, slow excitatory synaptic potentials could be recorded in some VIP-reactive neurones after extrinsic denervations were combined with removal of the myenteric plexus. Thus, it is unlikely that substance P alone mediates the slow excitatory synaptic potentials recorded in submucous neurones. In contrast, a few VIP-reactive terminals remained in some of the ganglia studied after the combined myectomy and extrinsic denervation (Figs 2*C* and 3*C*). This confirmed previous observations that not all VIP-reactive terminals degenerate after myectomies (Costa & Furness, 1983; Bornstein *et al.* 1987). Slow excitatory synaptic potentials were, however, observed in some VIP-reactive neurones in submucous ganglia containing no VIP-reactive terminals (Bornstein *et al.* 1987) so that it is unlikely that these responses are entirely mediated by VIP. It is possible that the slow excitatory synaptic potentials result from the action of a number of substances, perhaps including substance P and VIP.

The VIP-reactive neurones of the submucous plexus in guinea-pig small intestine project to the mucosa. There is considerable evidence that these neurones are non-cholinergic secretomotor neurones (e.g. Keast, 1987). The synaptic input to these neurones is complex, involving a number of different types of nerve terminal which arise from several sources. They receive cholinergic excitatory inputs from both myenteric and submucous neurones (Bornstein *et al.* 1987), inhibitory input from sympathetic and myenteric neurones and, in many cases, slow excitatory input from myenteric neurones (Bornstein *et al.* 1987). Some VIP-reactive neurones may also receive slow excitatory input from submucous neurones (Furness, Costa, Rokaeus, McDonald & Brooks, 1987). It seems likely that the significances of these inputs and the roles of these neurones will only be fully clarified when their responses to physiological stimuli can be examined.

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