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SYNAPTIC INPUTS TO IMMUNOHISTOCHEMICALLY IDENTIFIED NEURONES IN THE SUBMUCOUS PLEXUS OF THE GUINEA-PIG SMALL INTESTINE

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SUMMARY

1. Electrophysiological recordings were made from neurones in the submucous plexus of the guinea-pig small intestine, and these neurones were classified according to their synaptic inputs.

2. The neurones from which recording were made were filled during the recording period with the fluorescent dye, Lucifer Yellow, so they could be re-indentified after processing for immunohistochemical localization of vasoactive intestinal peptide (VIP).

3. The presence or absence of VIP-like immunoreactivity was determined for a total of 130 neurones whose synaptic inputs had been fully characterized and eighty-two were found to be VIP reactive. After the VIP reactivity had been assessed, the preparations were reprocessed to reveal immunoreactivity for neuropeptide Y (NPY) and a further twenty-three neurones (none of which were reactive for VIP) were found to be reactive for this peptide. Of the remaining twenty-five neurones, nineteen were not reactive for either VIP or NPY and six could not be re-identified after reprocessing.

4. Electrical stimulation of internodal strands evoked excitatory synaptic potentials lasting 20–30 ms (fast responses) in all but one of the 130 neurones studied.

5. Almost all the VIP-reactive neurones (seventy-eight of eighty-two cells) exhibited inhibitory synaptic potentials, ranging in amplitude from 2 to 30 mV and lasting 150–1500 ms, but few of the VIP-negative neurones had such responses (six of forty-eight cells). No inhibitory synaptic potentials could be evoked in any of the NPY-reactive neurones.

6. Most VIP-reactive neurones (sixty-nine) had a slow excitatory synaptic potential which could be evoked by a single stimulus, lasted 5–20 s and was associated with an increase in input resistance. Only one NPY-reactive neurone had a slow excitatory potential, but such potentials were seen in nine of the nineteen VIP-negative, NPY-negative neurones.

7. In nine of the twenty-three NPY-reactive neurones a single stimulus evoked an excitatory synaptic potential (intermediate excitatory synaptic potential) lasting 500–1500 ms and associated with a fall in the input resistance. None of the

VIP-negative, NPY-negative neurones exhibited the intermediate excitatory potentials but it was not possible to determine whether such potentials could be evoked in VIP-reactive neurones because the inhibitory synaptic potentials would obscure such events.

8. It is concluded that neurochemically distinct populations of submucous neurones can be distinguished physiologically on the basis of the differing combinations of types of synaptic input they receive.

INTRODUCTION

Histochemical studies of the submucous plexus have revealed a number of different peptide-containing neurones. The most complete of these studies was of the submucous plexus of the guinea-pig small intestine where neurones can be readily divided into two groups: those that are immunoreactive for vasoactive intestinal peptide (VIP), and those that are immunoreactive for the enzyme choline acetyltransferase (ChAT) (Furness, Costa & Keast, 1984). Neurones reactive for ChAT can be subdivided into three groups: a population reactive for ChAT alone; a population reactive for both ChAT and substance P; and a population reactive for ChAT and a number of peptides, namely neuropeptide Y (NPY), cholecystokinin, somatostatin and calcitonin gene-related peptide (Furness *et al.* 1984; Furness, Costa, Gibbins, Llewellyn-Smith & Oliver, 1985).

Electrophysiological studies have shown that neurones in the guinea-pig submucosa receive synaptic inputs from cholinergic neurones that, acting via nicotinic receptors, generate fast excitatory synaptic potentials (Hirst & McKirdy, 1975; Surprenant, 1984*a*). Some of these neurones also exhibit inhibitory synaptic potentials which are mimicked by ionophoretically applied noradrenaline and dopamine (Hirst & McKirdy, 1975; Hirst & Silinsky, 1976; Surprenant, 1984*a*; North & Surprenant, 1985). Slow excitatory synaptic potentials have been recorded in many of the submucous neurones with inhibitory inputs (Surprenant, 1984*a*). Surprenant (1984*b*) has also reported that there are some neurones in the submucous plexus which do not have any synaptic input.

The aim of this present study was to examine the synaptic input to neurochemically identified submucous neurones of the guinea-pig small intestine using a combination of electrophysiological and immunohistochemical techniques (Bornstein, Costa, Furness & Lees, 1984). Submucous neurones were impaled with micro-electrodes containing a mixture of the fluorescent dye, Lucifer Yellow, and KCl solution. Cells from which recordings were taken were filled with the dye so they could be re-identified after processing for immunohistochemical localization of either VIP or NPY. These two peptides were chosen because each gives an intense fluorescence after immunohistochemical processing and between them they mark about three-quarters of the submucous neurones; VIP is found in all such neurones that do not contain ChAT and NPY is found in over half those that do (Furness *et al.* 1984).

A brief account of some of these results has been published elsewhere (Bornstein, Furness & Costa, 1985).

METHODS

Guinea-pigs (200–350 g) were stunned and their carotid arteries and spinal cords severed. Preparations of submucous plexus were dissected from the small intestine using methods described by Hirst & McKirdy (1975). A segment of intestine 20–25 cm from the ileo-caecal junction was removed, cut along its mesenteric border and pinned flat with the mucosa uppermost in a dissecting dish. The mucosa was stripped from a region 6–15 mm long and 8–10 mm wide (the normal width of the whole open intestine was 12–18 mm) and the submucous plexus was then peeled away from the underlying circular muscle. The sheet of submucous plexus was transferred to a small organ bath in which it was stretched to its original dimensions and pinned with the mucosal surface down. Throughout the dissection and the subsequent recording period, the preparation was bathed in a modified Krebs solution (composition in mM; NaCl, 133; KCl, 4·7; $CaCl_2$, 2·8; $MgSO_4$, 0·6; NaH_2PO_4, 1·2; $NaCO_3$, 16·3; glucose, 8; bubbled with 95% O_2, 5% CO_2.

The electrophysiological procedures and methods used to fill cells with Lucifer Yellow were essentially the same as those described by Bornstein *et al.* (1984). The organ bath was mounted on the stage of a compound microscope modified to allow the preparation to be viewed with fluorescent light epi-illumination or with red light trans-illumination (Lees & Gray, 1982). In some later experiments (twelve preparations), an inverted microscope with an epifluorescence attachment was used. Micro-electrodes (resistance 180–350 MΩ) filled with a 0·5% (w/v) solution of Lucifer Yellow in 0·5 M-KCl were used to record from neurones and simultaneously to fill them with the fluorescent dye.

Synaptic inputs to the impaled neurones were activated via a focal glass micro-electrode (tip diameter 10–20 μm) filled with saline solution or a tungsten micro-electrode (tip 20 μm) in contact with an internodal strand or the surface of the ganglion. In most cases the tip diameter of the stimulating electrode was similar to, or larger than, the diameter of the internodal strand being stimulated so that all nerve fibres in the strand could be activated. The stimulus strength used to characterize the inputs to a neurone was always at least twice that required to evoke a minimal fast excitatory synaptic potential or an antidromic action potential from the internodal strand being stimulated.

Neurones were characterized on the basis of the synaptic potentials that could be evoked in them. The focal electrode was placed on as many different internodal strands as possible to ensure that all the axons arriving at the ganglion and impinging on a neurone were stimulated and a cell was said to lack a particular type of synaptic response only if stimulation of at least three different internodal strands failed to evoke it. In almost all cases, when inhibitory synaptic potentials or slow excitatory synaptic potentials were recorded (see Results), they could be evoked from all internodal strands entering a ganglion.

Once the different types of synaptic potentials that could be evoked in a neurone had been identified and the cell had been filled the recording electrode was withdrawn. The durations of impalements were kept to the minimum times necessary to characterize the neurones, both to maximize the number of cells that could be studied and to reduce the effects of prolonged impalement on the immunoreactivity of the cells (see Bornstein *et al.* 1984). After each impalement, the nerve cell body and its ganglion were then drawn and the position in relation to other filled cells was noted.

After each electrophysiological experiment, the preparation was processed for immunohistochemical localization of VIP (Costa, Buffa, Furness & Solcia, 1980). After fixation the preparation was dehydrated and rehydrated before being exposed for at least 12 h to an anti-VIP antibody (code 7913 from Dr J. H. Walsh) raised in a rabbit; it was then washed and exposed for 1 h to a second (anti-rabbit) antibody coupled to rhodamine (Cappel Laboratories). Use of rhodamine allowed the immunofluorescence to be distinguished from the Lucifer Yellow fluorescence in the filled cells (Reaves & Hayward, 1979).

After processing neurones were re-identified by their Lucifer Yellow fluorescence and the filters were then switched to show the VIP immunoreactivity. The experimenter monitoring the presence or absence of immunoreactivity had no prior knowledge of the electrophysiological properties of the neurone being examined. If a cell proved to be negative for VIP, the preparation was reprocessed to reveal any immunoreactivity for NPY using a primary antibody raised in a rabbit (code JBM 263/1 from Drs C. Maccarrone and B. Jarrott) and the same second antibody as before.

This was feasible because the NPY reaction was very intense and VIP and NPY are not in the same cells (Furness *et al.* 1984).

Measurements of the lengths of the long processes of filled cells were made immediately after the electrode had been withdrawn from the cell.

In some experiments where the cells' shapes were to be determined the micro-electrodes were filled with a 1 % solution of Lucifier Yellow in 0·01 % formaldehyde. This mixture appeared to fill the short processes of the neurones well without causing the slight swelling sometimes seen with the solution used for electrophysiology. In other experiments for this purpose the electrodes were filled with a 3 % solution of Lucifer Yellow in distilled water.

RESULTS

The synaptic inputs to 212 neurones in sixty-three preparations of submucous plexus were examined and in 164 cases fully characterized. The immunoreactivity for VIP was determined for 130 of the electrophysiologically identified neurones and it is this sample that is analysed below.

Electrophysiological identification

Five kinds of synaptic potential were identified on the basis of their duration, the number of stimuli required to evoke them and the direction of the potential change and of the associated change in input resistance. These were fast excitatory synaptic potentials, inhibitory synaptic potentials, slow excitatory synaptic potentials, intermediate excitatory synaptic potentials and 'multipulse' slow excitatory synaptic potentials.

All but one of the cells exhibited fast excitatory synaptic potentials (Fig. 1). These potentials had durations of 25–50 ms. In ten experiments the number of axons contributing to the maximal fast excitatory potential that could be evoked from a single internodal strand was estimated by counting the number of increments in the amplitude of the response as the stimulus strength was increased from zero to supramaximal. These estimates ranged from four to six with a mean of 5·5 axons per internodal strand. Hirst & McKirdy (1975) and Surprenant (1984*a*) have shown that fast excitatory synaptic potentials in submucous neurones are blocked by both tubocurarine and hexamethonium. Thus, the fast excitatory synaptic potentials are almost certainly the result of transmission from cholinergic neurones.

In eighty-four cells (65 %), inhibitory synaptic potentials, ranging in amplitude from 2 to 30 mV and with durations of 150–1500 ms, were observed (Fig. 2, see also Fig. 7*A*). The inhibitory synaptic potentials were seen immediately after the fast excitatory potentials evoked by the same stimulus and had a latency of 30–75 ms. An inhibitory synaptic potential could be evoked in some neurones by a single pulse (Fig. 2*A*) but in many others at least three pulses at 30 Hz were required to evoke a recognizable inhibitory response. The stimulus strength required to evoke inhibitory synaptic potentials varied between neurones, but it was never less than that required to evoke the lowest threshold fast excitatory potentials and usually less than that required to evoke maximal fast excitations. The inhibitory synaptic potentials were associated with substantial increases in membrane conductance (Figs. 2*B* and *C* and 3*A*), were enhanced by depolarization and were depressed by hyperpolarization as has been described by other workers (Hirst & McKirdy, 1975; Surprenant, 1984*a*; North & Surprenant, 1985). As was previously reported by Surprenant (1984*a*), the

dependence of the amplitude of the inhibitory response on the number of stimuli was often very marked with one pulse giving no hyperpolarization, two pulses giving a submaximal response and three to five pulses giving a maximal inhibitory potential (Fig. 2*C*). Amplitude was also dependent on the frequency of stimulation: three pulses at 10 Hz usually produced a smaller response than the same number of stimuli at 30 Hz.

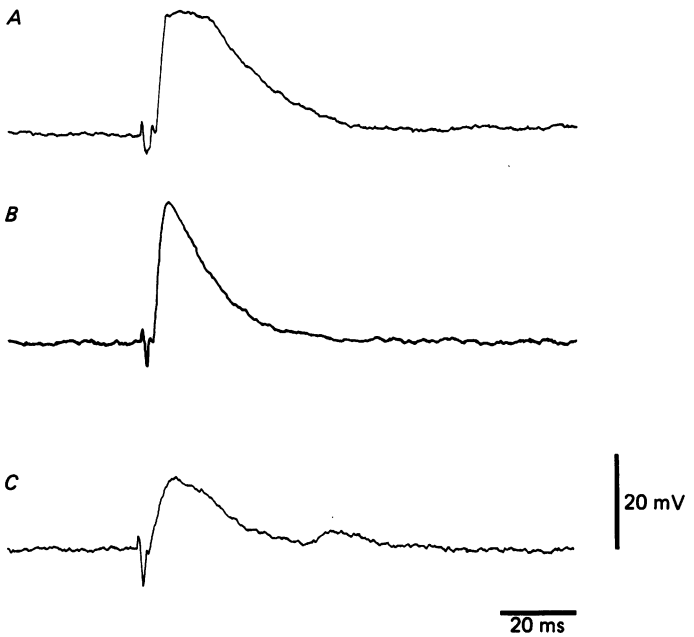

Fig. 1. Fast excitatory synaptic potentials recorded in a VIP-reactive neurone (*A*), an NPY-reactive neurone (*B*) (that shown in Fig. 8) and a VIP-, NPY-negative neurone (*C*). These responses were evoked by submaximal stimuli but in each case two or more presynaptic fibres were activated.

Slow excitatory synaptic potentials were seen in many cells (seventy-nine; 61 %). These were evoked by a single stimulus, lasted 5–20 s and were associated with a decrease in membrane conductance (Fig. 3*A*). The latency of the slow excitatory responses was 50–100 ms in the absence of a preceding inhibitory synaptic potential and the amplitude depended on the number of stimuli. The stimulus strength required to evoke a slow excitatory potential was usually similar to, or slightly less than, that required to evoke the inhibitory synaptic potentials in the same neurone. The slow excitatory potentials were enhanced by depolarization and depressed by hyperpolarization (Fig. 3*B*). Similar slow excitations have been described by Surprenant (1984*a*), who reported that they were due to a decrease in the membrane permeability to potassium ions. Surprenant (1984*a*) also reported that the slow excitatory potentials are largely confined to neurones with inhibitory synaptic potentials and this was confirmed in this present study (see Table 1).

In some cells lacking slow excitatory responses and inhibitory synaptic potentials, another type of synaptic potential was observed, the intermediate excitatory

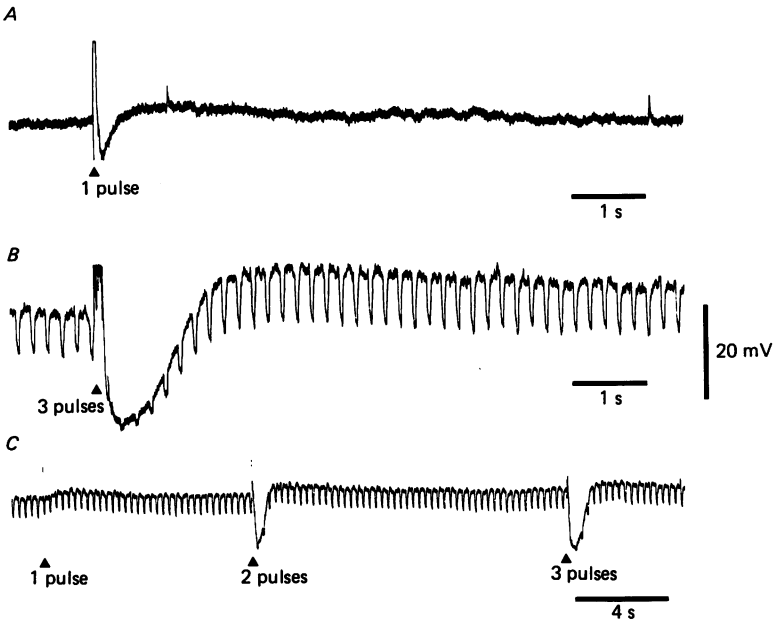

Fig. 2. Inhibitory synaptic potentials evoked in three different VIP-reactive neurones by one pulse (*A*), three pulses at 30 Hz (*B*) and one, two and three pulses (*C*). The records shown in *B* and *C* were obtained while hyperpolarizing current pulses were being injected into the cells so that the size of the resulting hyperpolarization provided an estimate of the input resistance of the cell. In this and subsequent Figures the action potentials evoked by the stimuli are attenuated by the low frequency response of the chart recorder used to make the records. Records retouched.

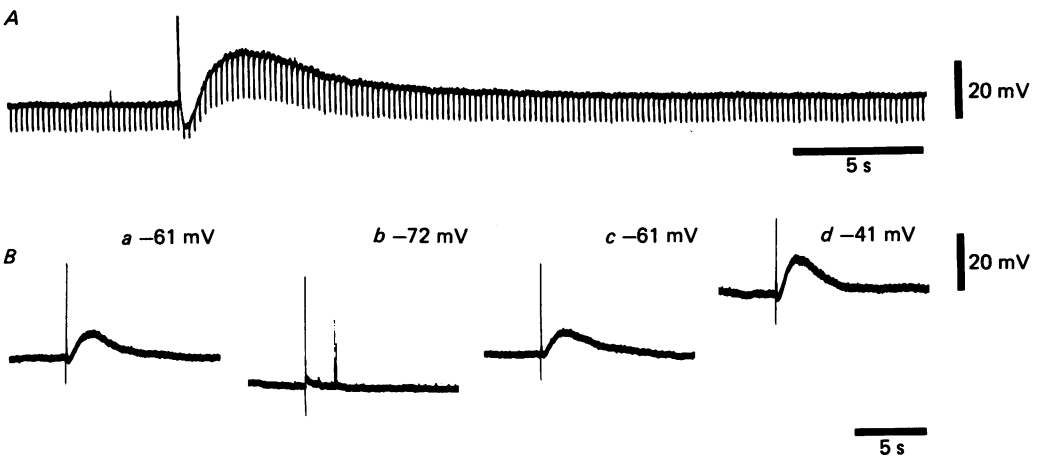

Fig. 3. Slow excitatory synaptic potentials evoked in a VIP-reactive neurone. *A*, hyperpolarizing current pulses were passed through the recording electrode to monitor the cell's input resistance during a slow excitatory potential evoked by two pulses at 30 Hz. *B*, slow excitatory potentials evoked in the same cell by single stimuli at the resting membrane potential of 61 mV (*a* and *c*), with the membrane potential hyperpolarized by 11 mV (*b*) and depolarized by 20 mV (*d*). Records retouched.

synaptic potential (Fig. 4). These were evoked by one to three stimuli at 30 Hz, had a latency of 30–50 ms and lasted only 0·5–2 s (Fig. 4*A* and *B*). The stimulus strength required to evoke this response was similar to that required to evoke the fast excitatory potentials in the same neurones. This response was associated with an

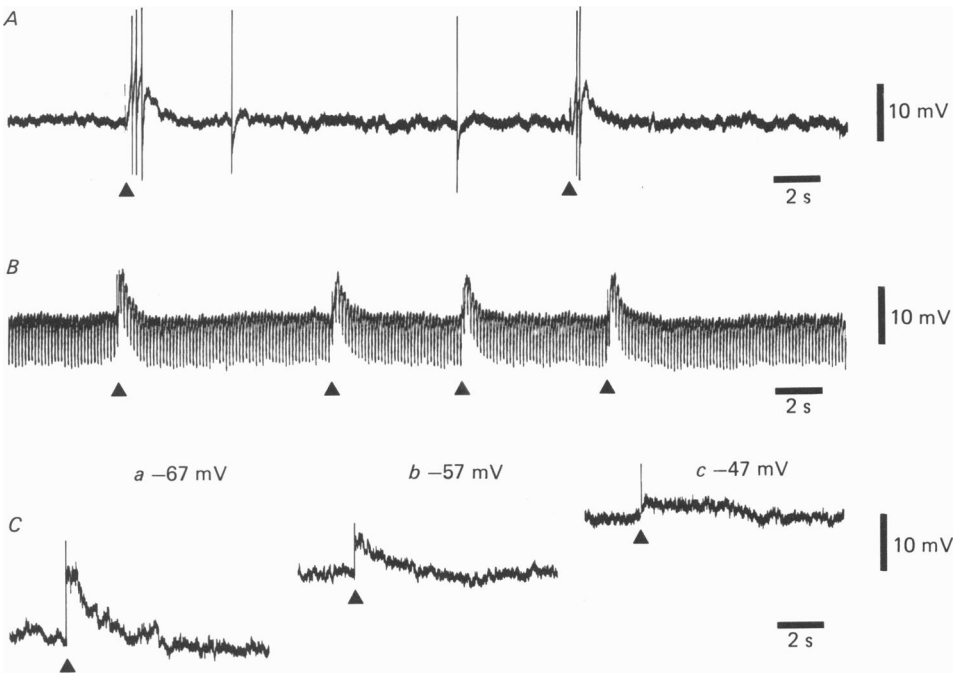

Fig. 4. *A*, intermediate excitatory synaptic potentials evoked by single focal stimuli in an NPY-reactive neurone; note the action potentials that were triggered by each stimulus. Two spontaneous action potentials were also observed between the stimuli. The reduction in input resistance associated with this response is illustrated in *B*. *C* compares a response recorded in this cell at the resting potential of −57 mV with those recorded after hyperpolarizing to −67 mV (*a*) and depolarizing to −47 mV (*C*). Records retouched.

increase in membrane conductance (Fig. 4*B*); it was enhanced by hyperpolarization and depressed by depolarization (Fig. 4*C*). Intermediate excitatory potentials were seen in nine neurones; since, however, the inhibitory synaptic potential can obscure this response, it may be that many more neurones actually receive this type of input.

A multipulse slow excitatory synaptic potential was observed in eight neurones when an internodal strand was stimulated for 2 s at 20 Hz (Fig. 5). At least ten pulses were required to evoke these potentials which were usually small (2–4 mV), lasted 10–30 s and were associated with a conductance decrease (Fig. 5*C*). Such potentials could, however, sometimes trigger action potentials (Fig. 5*B*). The stimulus strength required to evoke the multipulse responses was usually equal to, or greater than, that required to evoke maximal fast excitatory potentials from the same internodal strands.

A different pattern of synaptic response was seen in eight neurones. Stimulation

with one to five pulses at 10–30 Hz sometimes evoked fast excitatory synaptic potentials which were not stimulus locked, but occurred up to 30 s after the stimulus (Fig. 6, late fast excitatory synaptic potentials). In contrast to the other synaptic potentials observed in these experiments the late fast excitatory synaptic potentials

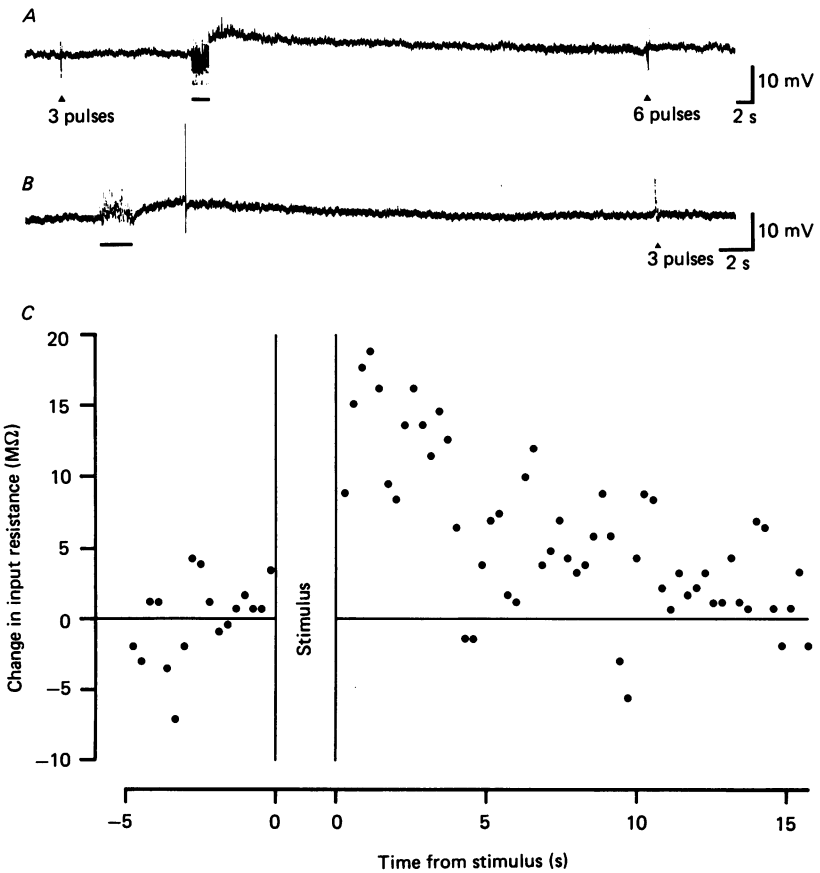

Fig. 5. Multipulse slow excitatory synaptic potentials in two different VIP-, NPY-negative neurones. *A*, the effects of three and six pulses at 30 Hz are compared with those of 20 Hz stimulation for 2 s (bar); *B*, the effects of three pulses at 30 Hz are compared with those of 20 Hz for 2 s (bar). Records retouched. *C*, the changes in input resistance produced in the neurone of *B* by 2 s of 20 Hz stimulation are shown; each point was calculated by taking the mean of four successive estimates of the change in input resistance from the original record; the first two of these estimates were then discarded and two subsequent estimates were included to give the next sample of four. This method of smoothing was followed because the individual measurements were significantly influenced by base-line noise.

were normally evoked from only one internodal strand. In five of these eight cells, spontaneous fast excitatory synaptic potentials could also be observed. Many other cells (thirty-eight) with spontaneous fast excitatory synaptic potentials did not exhibit the late fast responses.

Correlations with immunoreactivity

Eighty-two of the sample of 130 electrophysiologically identified neurones examined for VIP immunoreactivity were positive for this peptide (as shown in Fig. 7). Of those which were VIP negative, twenty-three showed immunoreactivity for NPY (e.g. Fig. 8), nineteen were not reactive for either VIP or NPY, and the other six

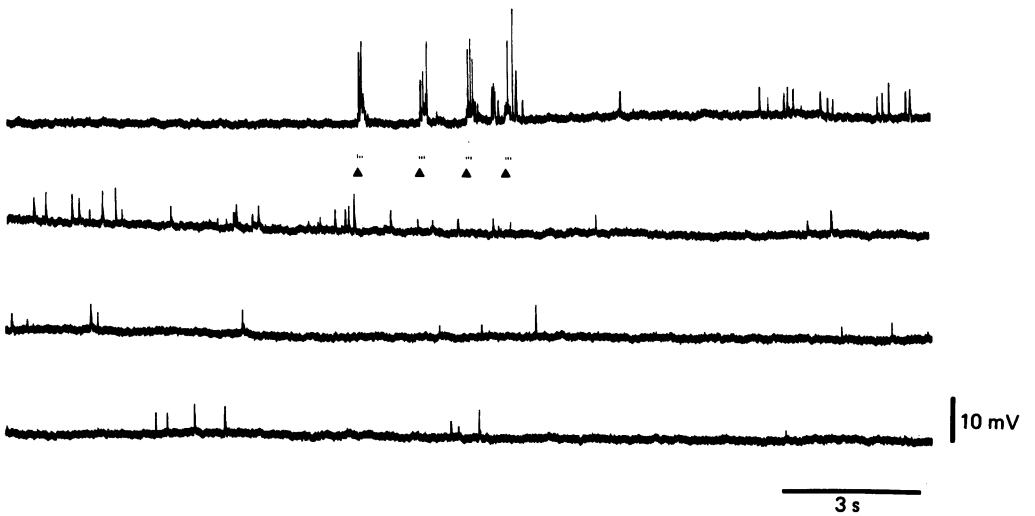

Fig. 6. A continuous record showing the effects of four bursts of stimuli (arrows) each of three pulses at 30 Hz on a VIP-reactive neurone, the frequency of 'spontaneous' fast excitatory synaptic potentials was greatly enhanced in the 20–30 s following the stimuli. A small slow excitatory synaptic potential was also evoked and can be seen in the first two lines. Record retouched.

could not be found again after processing for NPY. No differences in either resting membrane potential or input resistance were observed between VIP-positive and VIP-negative cells. Fast excitatory synaptic potentials were evoked in each histochemically identified type of neurone; the responses shown in Fig. 1 were recorded in a VIP-reactive cell (*A*), an NPY-reactive cell (*B*) and a cell that did not react for either peptide (*C*). No fast excitatory synaptic potential could be evoked in one neurone that was VIP negative but could not be re-identified after processing for NPY reactivity.

Marked differences in the proportions of the histochemically defined cell types exhibiting inhibitory synaptic potentials and slow excitatory synaptic potentials were found (Table 1). All but four of the VIP-positive neurones exhibited inhibitory responses; the inhibitory synaptic potentials shown in Fig. 2 were all recorded in VIP-reactive neurones. Only six of the forty-eight VIP-negative neurones had such responses, but none of these were NPY positive. Similarly, slow excitatory potentials were seen in sixty-nine of the eighty-two VIP-positive neurones (e.g. Figs. 3 and 7*A*) but only one of the twenty-three NPY-positive neurones had a comparable response. Thus, the presence of either an inhibitory synaptic potential or a slow excitatory

TABLE 1. Correlation of neurochemical cell type with synaptic input

Neurochemical type	Number of cells	Number of neurones with a given synaptic cell response			
		Fast e.s.p.s	I.s.p.s	Slow e.s.p.s	I.s.p.s plus slow e.s.p.s
VIP reactive	82	82	78	69	67
VIP negative	48	47	6	9	4
NPY reactive	23	23	0	1	0
VIP, NPY negative	19	18	6	9	4

Excitatory synaptic potential, e.s.p.; inhibitory synaptic potential, i.s.p.

synaptic potential was highly correlated with the presence of VIP-like immunoreactivity in submucous neurones. Neurones reactive for NPY, however, rarely had such synaptic inputs. Neurones that were not reactive for either VIP or NPY were a mixed group with some showing responses typical of VIP-positive neurones and others exhibiting only fast excitatory synaptic potentials.

NPY-reactive neurones occasionally exhibited intermediate excitatory potentials (eight neurones), multipulse slow excitatory synaptic responses (four neurones) or

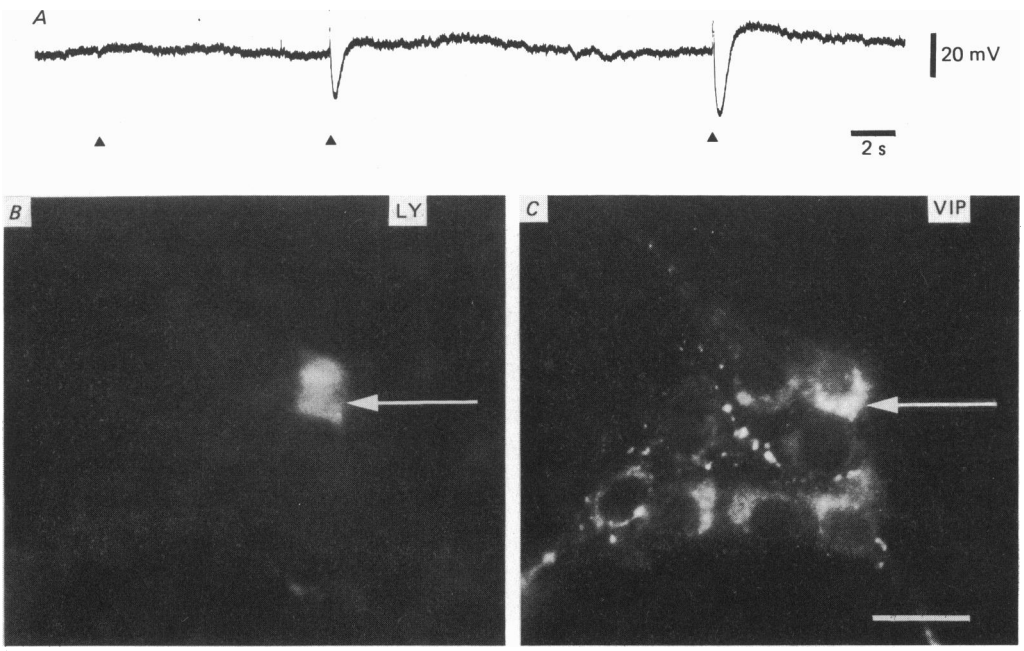

Fig. 7. *A*, inhibitory synaptic potentials and slow excitatory synaptic potentials evoked in a submucous neurone by one, two and three stimuli (arrows) at 30 Hz (left to right). *B*, a micrograph showing this neurone after it was filled with Lucifer Yellow (LY, arrow). *C*, a micrograph showing the ganglion containing this neurone (arrow) after processing for VIP-like immunoreactivity; note, the Lucifer Yellow-filled cell was also reactive for VIP. Calibration, 25 μm.

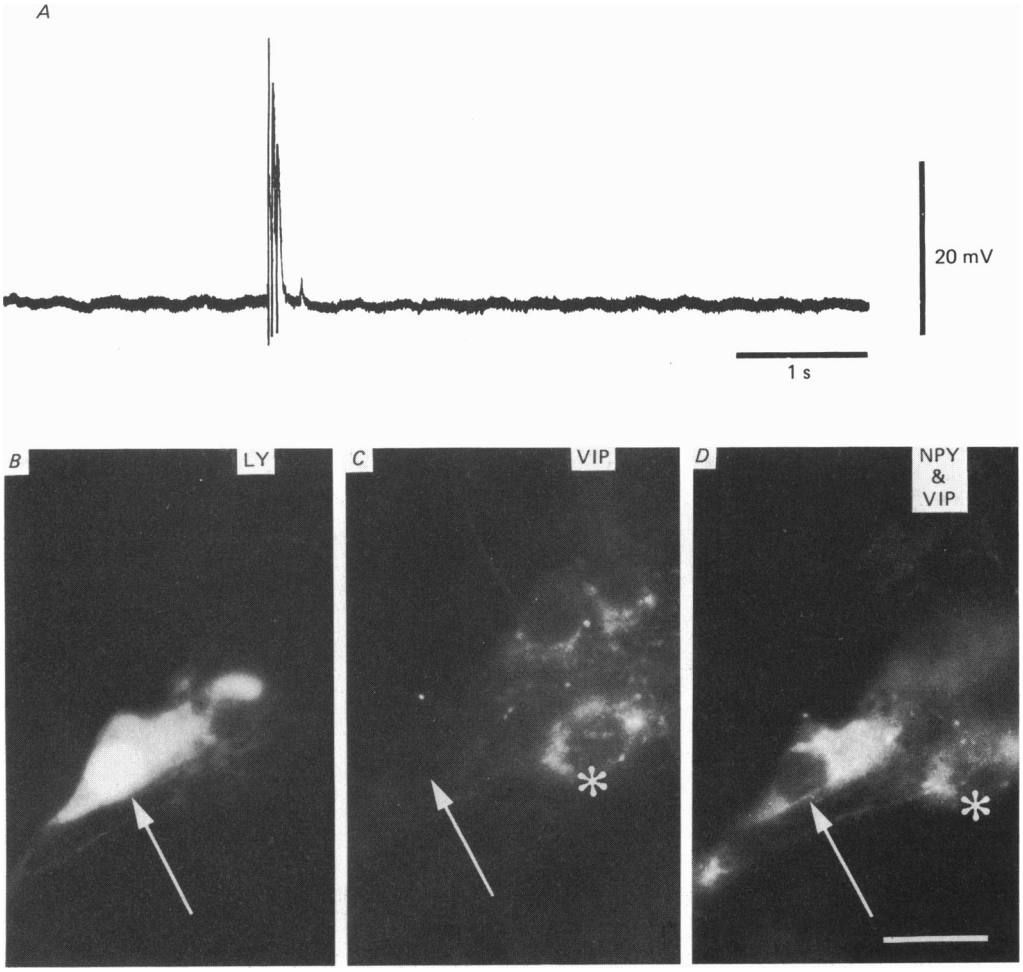

Fig. 8. *A*, three fast excitatory synaptic potentials evoked by three stimuli at 30 Hz in a Lucifer Yellow (LY)-filled neurone (*B*, arrow): neither inhibitory synaptic potentials nor slow excitatory synaptic potentials could be evoked in this cell which was VIP negative (*C*) but NPY positive (*D*). Other neurones in the ganglion are reactive for VIP; one of these is marked by an asterisk in *C* and *D*. Note that the level of focus differs slightly between *C* and *D*. Calibration, 25 μm.

both (one neurone). Intermediate excitatory potentials were not seen in any of the neurones which were negative for both VIP and NPY, but three of these cells did exhibit multiple excitatory synaptic potentials. The difference in the proportions of NPY-positive neurones and neurones negative for VIP and NPY with intermediate excitatory responses was significant ($P < 0{\cdot}01$, χ^2 test) which suggests that the presence of such responses is correlated with the presence of NPY in the neurone.

Late fast excitatory synaptic potentials were observed in both VIP-positive neurones (six cells) and NPY-positive neurones (two cells).

Nine neurone pairs in these preparations were found to be dye coupled. These neurones were recognized when impalements were made in ganglia that had no

previously filled cells. When the dye was injected two cells became fluorescent simultaneously and with about the same intensity (Fig. 9). The processes of these cell pairs were usually equally well filled. The dye-coupled cells had inhibitory synaptic potentials and in most cases slow excitatory potentials. They were all VIP positive.

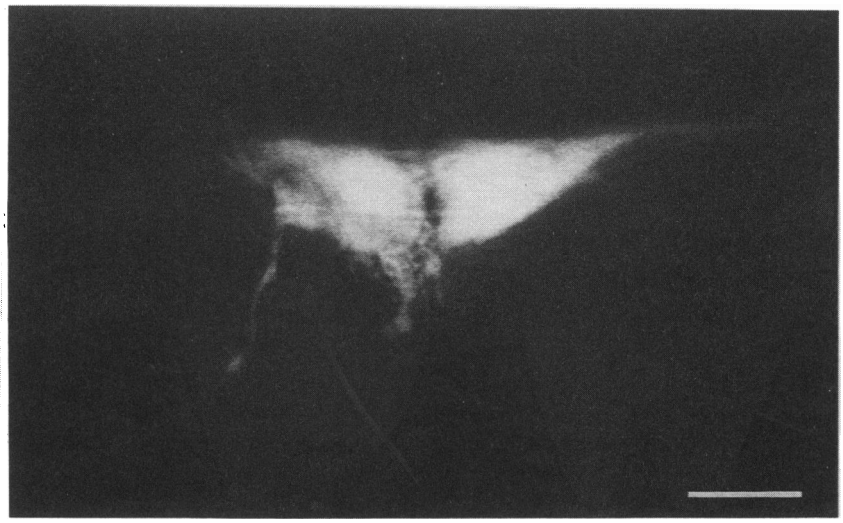

Fig. 9. A dye-coupled cell pair filled with Lucifer Yellow; the cell on the right was the one impaled and exhibited fast and slow excitatory synaptic potentials and inhibitory synaptic potentials. Both cells were immunoreactive for VIP. Calibration, 25 μm.

Projections and shapes of filled neurones

The long processes of the neurones with inhibitory potentials were followed in the submucous plexus. In some cases the distance between a cell body and the point at which its process could no longer be detected was up to 1500 μm and the actual length of the process as it followed an irregular course via internodal strands from ganglion to ganglion was substantially greater. Most neurones impaled had only one long process (defined as a process which left the ganglion in which its cell body lay) and only a minority of these branched before either fading to the point of being unrecognizable or ending in a bright expansion at the surface of the preparation from which the mucosa had been removed. The bright expansions probably represented resealed cut fibres (retraction bulbs) similar to those observed in the myenteric plexus by Erde, Sherman & Gershon (1985). The distances between the cell bodies and the points of disappearance of their longest processes were measured for fifty-nine neurones of which forty-one had inhibitory synaptic potentials and the results are shown in Table 2. Neurones with inhibitory responses projected an average of 690 μm, those without such responses averaged only 420 μm. As might be expected, when the lengths of VIP-reactive cells were compared to those of VIP-negative cells a similar difference was found. A relatively small number of NPY-reactive neurones could be examined in this way and so two preparations were stained for NPY without electrophysiological identification and immunoreactive processes were traced as

described by Furness *et al.* (1985). It was found that the long processes of NPY-reactive neurones averaged 290 μm (Table 2), less than half the length of the processes of the VIP-reactive cells (690 μm). Neither the VIP-reactive neurones examined after being filled with Lucifer Yellow nor the NPY-reactive neurones traced using histochemical methods alone showed a preference for an oral, anal or circumferential direction of projection.

TABLE 2. Distances between cell bodies and the 'ends' of long processes

Electrophysiological neurone type	Number of cells	Mean distance ± standard error (μm)
Inhibitory response	41	690 ± 54
No inhibitory response	18	420 ± 49
Neurochemical type		
VIP reactive	39	690 ± 53
VIP negative	16	380 ± 46
NPY reactive (filled)	8	320 ± 60
NPY reactive (unfilled)	50	290 ± 19

The shapes of NPY-reactive neurones in the submucous plexus can be clearly distinguished in histochemical preparations and have been described elsewhere (Furness, Costa, Emson, Håkanson, Moghimzadeh, Sundler, Taylor & Chance, 1983; Furness *et al.* 1985). The reactivity in VIP-positive neurones is, however, normally found around the nucleus and does not fill the cytoplasm (e.g. Fig. 7*C*; Pls. 19–22 of Costa & Furness, 1983), so that the cell shape cannot be determined from histochemistry alone. Accordingly, sixty neurones were filled with Lucifer Yellow without regard to their electrophysiological properties; these cells were then processed for peptide reactivity and their shapes were drawn. Forty-eight of the filled neurones were reactive for VIP, four were reactive for NPY, five were VIP, NPY negative and three could not be adequately identified after processing for immunohistochemistry. Examples of these cells are shown in Fig. 10 which shows six VIP-reactive neurones, two NPY-reactive neurones and two VIP-, NPY-negative neurones. All the neurones in the submucous plexus appeared to be of the same morphological type, Dogiel Type III (Dogiel, 1899).

DISCUSSION

The results described here show a strong correlation between the peptide content of a submucous neurone and its synaptic inputs. Virtually all the neurones exhibited fast excitatory synaptic potentials but there were marked differences in the incidence of other types of synaptic potentials between the different neurochemical classes of neurone. Seventy-eight of the eighty-two VIP-reactive neurones (95 %) had inhibitory synaptic potentials. None of the NPY-reactive neurones exhibited such responses. Slow excitatory synaptic potentials were recorded in sixty-nine (84 %) of the VIP-positive cells but similar responses were seen in only one of the twenty-three NPY-reactive neurones. Many of the NPY-reactive neurones had intermediate

Fig. 10. Examples of the shapes of submucous neurones drawn after filling with Lucifer Yellow and processing for immunohistochemistry. Cells *A* and *F* were negative for both VIP and NPY, cells *C*, *E*, *G*, *H*, *I* and *J* were VIP reactive and cells *B* and *D* were reactive for NPY (cell *B* was drawn from the immunofluorescent outline alone). Cells *E* were a dye-coupled pair. Calibration, 20 μm.

excitatory synaptic potentials, responses which were absent from those neurones that were negative for both VIP and NPY. VIP-reactive neurones may receive input from nerve fibres responsible for intermediate excitatory potentials but such responses could not be reliably detected in these neurones because of the presence of inhibitory and slow excitatory synaptic potentials.

The proportions of VIP- and NPY-reactive submucous neurones seen in this study differed markedly from those estimated from immunohistochemical studies. Thus, eighty-two of 130 cells (63 %) were immunoreactive for VIP after electrophysiological characterization but Furness *et al.* (1984) found, using much larger samples,

that only 45% (range 43–47%; 1370 neurones in four preparations) of submucous neurones were immunoreactive for VIP. Similarly, of the 124 neurones characterized for NPY, twenty-three (19%) were positive in the present study, but Furness *et al.* (1984) reported that 29% of their sample were NPY reactive. Differences such as these suggest that any examination of submucous plexus neurones based solely on electrophysiological studies may be subject to biased sampling. VIP-reactive neurones are usually found grouped together in the centre of submucous ganglia while ChAT-reactive neurones (including the NPY-reactive cells) although they are more numerous tend to be more peripheral (Furness *et al.* 1984). Thus, the VIP-reactive neurones may be encountered more often.

It is possible that all VIP-reactive neurones have inhibitory synaptic input and that only these neurones receive such inputs. The arrangement of the recording electrode and the focal stimulating electrode was such that it was not always feasible to place the stimulating electrode on each of the internodal strands running to a ganglion. So, in some cases, a few presynaptic fibres impinging on a neurone may have been left unstimulated. Furthermore, some of the VIP-negative neurones which had inhibitory potentials may have been false negatives, in that these cells actually contained VIP but impalement had led to a reduction in the immunoreactivity, as was seen in a similar study of enkephalin neurones in the myenteric plexus (Bornstein *et al.* 1984), thus causing the cell to be misclassified.

Pharmacological studies of the inhibitory synaptic potentials suggest that they arise from activation of adrenoceptors (Hirst & Silinsky, 1975; Surprenant, 1984*a*; North & Surprenant, 1985). Moreover, they can be abolished by agents that block catecholamine release from noradrenergic terminals (Hirst & McKirdy, 1975; North & Surprenant, 1985). Noradrenergic terminals, identified by immunoreactivity for dopamine-β-hydroxylase, preferentially innervate the central regions of submucous ganglia (Costa & Furness, 1984), the region in which most VIP-reactive neurones are found (Furness *et al.* 1984). Hirst & McKirdy (1975) found, however, that inhibitory responses could still be evoked in submucous ganglia from segments of intestine that had been extrinsically denervated, which suggests that at least a component of these responses is intrinsic in origin. There are no intrinsic adrenergic or dopaminergic neurones in the guinea-pig small intestine (Costa & Furness, 1971; Furness, Costa & Freeman, 1979; Howe, Provis, Furness, Costa & Chalmers, 1981). It may be that the inhibitory responses result from activation of two types of axons: a noradrenergic population arising from sympathetic ganglia, and another population intrinsic to the intestine and of unknown neurochemistry.

Little evidence is available about the transmitters responsible for the other types of synaptic potential observed in the present study. Immunohistochemical studies have identified many different putative neurotransmitters within nerve terminals in submucous ganglia; these include substance P, VIP, somatostatin, gastrin-releasing peptide and 5-hydroxytryptamine (Furness, Llewellyn-Smith, Bornstein & Costa, 1986). Substance P and 5-hydroxytryptamine each depolarize submucous neurones via conductance changes similar to those associated with the slow and the intermediate excitatory synaptic potentials respectively (Hirst & Silinsky, 1975; Surprenant, 1984*a*), but the actions of the other possible transmitters within this preparation have not been determined.

The two different neurochemical classes of neurones, whose synaptic inputs have been classified in this study, probably play major roles in the control of intestinal secretion and absorption. Although NPY has been used to locate one of these populations, these neurones also contain cholecystokinin (CCK), ChAT, somatostatin (SOM), and calcitonin gene-related peptide (CGRP). The roles of the different substances found in the CCK/CGRP/ChAT/NPY/SOM neurones are not known, although the presence of ChAT suggests that they are cholinergic. The processes of NPY-reactive neurones have been traced directly to the mucosa in immunohistochemical studies (Furness *et al.* 1985) and studies combining immunohistochemistry and lesions of neuronal pathways have shows that most, if not all, VIP-reactive neurones also project to the mucosa (Costa & Furness, 1983; Keast, Furness & Costa, 1984). Secretion from the mucosa of the guinea-pig small intestine in response to nerve stimulation, *in vitro*, appears to result from the release of acetylcholine and other stimulants (Cooke, 1984; Keast, Furness & Costa, 1985). The CCK/CGRP/ChAT/NPY/SOM submucous neurones probably represent at least half of the cholinergic secretomotor neurones (Furness *et al.* 1984). VIP has been found to be a potent stimulator of intestinal secretion (Gaginella, Hubel & O'Dorisio, 1982), and as the VIP-reactive cells are the only population of submucous neurones that do not contain ChAT (Furness *et al.* 1984) they are the prime candidates for the non-cholinergic secretomotor neurones. VIP-reactive neurones presumably act over a greater distance than the NPY-reactive neurones as they have longer projections (700 and 300 μm, respectively) but both are short by comparison with inhibitory motoneurones to the circular muscle, some of which project 20–30 mm anally (Bornstein, Costa, Furness & Lang, 1986). Neither the VIP-reactive nor the NPY-reactive secretomotor neurones show any preferential direction of projection. Thus, if there is any polarity of secretomotor reflexes, there would need to be a polarity of neurones impinging on these final neurones in the pathway.

The NPY-reactive neurones receive input primarily from cholinergic axons acting via nicotinic receptors. In contrast, the VIP-reactive neurones receive input from at least three different types of axon suggesting that they act to integrate input from a variety of sources including the sympathetic pathways which may provide a substantial component of the inhibitory input to these neurones (North & Surprenant, 1985).

The results described here provide further evidence for the idea that the histochemical coding of enteric neurones divides them into different functional groups (see also Furness & Costa, 1980; Bornstein *et al.* 1984).

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