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ELECTROPHYSIOLOGICAL CHARACTERISTICS DISTINGUISH THREE CLASSES OF NEURON IN SUBMUCOSAL GANGLIA OF THE GUINEA-PIG DISTAL COLON

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Abstract—Intracellular recordings were made from neurons in the submucosal ganglia of the guinea-pig distal colon. The recording electrode contained the intracellular marker biocytin, which was injected into neurons so that their electrophysiological characteristics could be correlated with their shape. Correlations of electrophysiology and shape have not been reported previously for neurons in this region. Three types of neuron were identified on electrophysiological grounds. Neurons of the first type (S neurons) had tetrodotoxin-sensitive soma action potentials, and received fast and slow excitatory synaptic inputs. They had uniaxonal morphologies and may function as secretomotor or possibly vasomotor neurons. The second type (AH neurons) received only slow synaptic input, while the soma action potential had tetrodotoxin-sensitive and -insensitive components with a shoulder on the falling phase and a long-lasting afterhyperpolarisation of the membrane potential following a single action potential. Neurons of this type had multipolar morphologies and provided dense innervation of adjacent submucosal ganglia. These neurons are similar to the submucosal intrinsic primary afferent neurons of the guinea-pig small intestine. The final type of neuron [the low-threshold (LT) neuron] had electrophysiological characteristics that set it apart from those described previously within enteric plexuses. They expressed tetrodotoxin-insensitive voltage-gated soma currents, did not have long-lasting afterhyperpolarisations and received only slow synaptic input. In addition, these neurons were very excitable: they had large input resistances and low thresholds for action potential discharge, and often fired action potentials in the absence of stimulation. Neurons with these characteristics were uniaxonal and thus are likely to be secretomotor or possibly vasomotor neurons.

This study has shown that submucosal neurons of the distal colon fall into three distinct types, which can be distinguished by a combination of electrophysiological and morphological criteria. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: enteric nervous system, autonomic ganglia, tetrodotoxin, neurophysiology, voltage-gated current.

The enteric nervous system contains the neural elements of reflex arcs that co-ordinate motility reflexes and modulate secretomotor and vasomotor activities of the mucosa.[9,17,45] Many of the neurons that are involved in secretomotor and vasomotor reflexes are in the submucosal ganglia.[8,18,26,43]

Intracellular recordings from enteric neurons using microelectrodes containing marker substances such as biocytin or neurobiotin have greatly advanced our understanding of the functional organisation of the enteric nervous system by allowing correlation of electrophysiological characteristics with neural shapes and projections, particularly within the myenteric plexus. Since the first intracellular recordings from myenteric and submucosal neurons,[21,23,39] investigators have classified enteric neurons according to their membrane properties and by the synaptic inputs that they receive. Neurons in the guinea-pig small intestine that receive fast synaptic input are called S neurons, using the terminology of Hirst *et al*.[21] Recordings from S neurons with microelectrodes containing marker substances have shown these neurons to have uniaxonal morphologies,[4,7,14,24,35] which has led investigators to the conclusion, based on the projections of their axons, that S neurons function as interneurons and motor neurons within enteric circuits.

Neurons in the guinea-pig small intestine that do not receive prominent fast synaptic input and have a prolonged (>2 s) hyperpolarisation of the membrane potential following single action potentials, the late afterhyperpolarising potential (late AHP), are designated

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Abbreviations: AH, designation of neurons with action potentials having inflections on their falling phase, followed by prolonged AHPs; AHP, afterhyperpolarising potential; EPSP, excitatory postsynaptic potential; I_A, A-type potassium current; I_H, hyperpolarisation-activated cation current; IPAN, intrinsic primary afferent neuron; LT, low-threshold (neurons); R_{in}, input resistance; S, designation of neurons receiving fast EPSPs and having action potentials with monophasic repolarisation; TTX, tetrodotoxin.

AH neurons.[21] When neurons that display AH-type electrophysiological characteristics are filled with marker substances, it is observed that these neurons have smooth oval cell bodies with several fine processes emanating from the cell body, a morphology defined by Dogiel[12] as type II.[3,6,7,13,14,24,25,35,37] These neurons reside in both plexuses and project to other enteric ganglia and to the mucosa, which led Dogiel[12] to speculate that they may serve a sensory function within the enteric nervous system. Since Dogiel's time, controversy has reigned as to whether the intestines might contain sensory neurons, and it is only in the past 10 years that direct physiological evidence has been obtained to support his original hypothesis (see Ref. 19).

Previous electrophysiological studies of the neurons in the submucosa of the colon, 10–20 cm distal to the colonic flexure, have revealed the presence of S- and AH-type neurons, although they were designated type 1 and type 2.[15] Type 3 and type 4 neurons were also identified. Type 3 neurons did not fire action potentials in response to depolarisation of the membrane, whereas type 4 neurons had low excitability; they only fired single action potentials in response to depolarising current. We have recently used immunohistochemical methods to investigate the types of neuron present in the submucosal ganglia of the guinea-pig distal colon in the 10 cm before the pelvic brim (30–40 cm distal to the colonic flexure)[34] and have identified four types: three types of secretomotor/vasomotor neuron and a class of intrinsic primary afferent neuron (IPANs). These four types of neuron correspond to the four types identified previously in the small intestine,[18,26] where the secretomotor/vasodilator neurons are S neurons and the IPANs are AH neurons.[2,17,18] In the present study, we have examined the same region of guinea-pig distal colon that was studied histochemically, using biocytin-filled microelectrodes in order to relate the electrophysiological characteristics of neurons in the submucosal ganglia with their morphologies.

EXPERIMENTAL PROCEDURES

Guinea-pigs (from the Anatomy and Cell Biology inbred Dunkin–Hartley colony) weighing 160–300 g were killed by being stunned and having their carotid arteries and spinal cords severed. All efforts were made to minimise animal suffering and the numbers of animals used, and conformed to National Health and Medical Research Council of Australia guidelines. They were approved by the University of Melbourne Animal Experimentation Ethics Committee. Segments of distal colon (2–3 cm) were taken between 2 and 5 cm oral to the pelvic brim, the oral end was marked, and the segments were placed in physiological saline (composition in mM: NaCl 118, KCl 4.8, $NaHCO_3$ 25, NaH_2PO_4 1.0, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 2.5; equilibrated with 95% O_2/5% CO_2) and initially kept at room temperature. The segment of colon was opened along its mesenteric border and pinned flat, mucosa uppermost, in a dissecting dish lined with silastic elastomer. The mucosa was gently removed from the underlying submucosa using fine forceps and the preparation flipped over and repinned so that the serosa was uppermost. The serosa and external musculature were then removed to yield a preparation of submucosa. The oral right-hand corner of the preparation was marked with a cut in anticipation of later morphological analysis, and the preparation was transferred to a small (volume 4 ml) silastic elastomer-lined recording dish and pinned flat with the surface that had faced the mucosa downwards. The recording dish was then transferred to the stage of an inverted microscope and continuously superfused with physiological saline that had been preheated to yield a bath temperature of 35°C. The tissue was equilibrated with perfusate for 1 h before recording commenced.

Neurons were impaled with conventional borosilicate glass microelectrodes filled with 2% biocytin (Sigma Chemicals) in 1 M KCl.[6,35] Electrode resistances were 100–210 MΩ. Recordings of membrane potential were made using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA). Signals were digitised at 1–10 kHz and stored using PC-based data acquisition software (Axoscope 1.0, Axon Instruments). Measurement of electrophysiological variables and curve fitting were done offline using Axoscope 1.0 and Origin 5.0 (MicroCal Software, Northampton, MA, USA).

Measurements of neuronal electrical properties were made after allowing the impalements to stabilise for 15 min, without applying intracellular holding current. At this time, the ability of the cell to fire an action potential upon intracellular current injection was assessed. Only neurons that were able to fire an action potential and had resting membrane potentials more negative than −40 mV were included in the electrophysiological analyses.

Excitability was assessed by injecting 500-ms depolarising current pulses of increasing intensity to reveal the maximum number and the period of firing of trains of action potentials.[31] Neurons that fired action potentials for less than half the duration of the imposed depolarisation (<250 ms), most of which fired for <50 ms, are described as being in a rapidly accommodating state, while those that fired for more than 250 ms are described as being in a slowly accommodating state.[31]

In order to test whether neurons received synaptic inputs, electrical stimuli were delivered to internodal strands using sharpened monopolar electrodes made from tungsten wire (10–50 μm tip diameter), insulated except at the tip. Stimulus pulses of 0.3 ms duration and 5–15 V intensity were delivered via a Grass S44 stimulator (Grass Medical Instruments, Quincy, MA, USA) controlled by a Master-8 programmable pulse generator (AMPI, Jerusalem, Israel). For analysis of fast excitatory postsynaptic potential (EPSP) amplitudes, we measured the amplitude of the largest fast EPSP that did not evoke an action potential at the resting membrane potential. Trains of pulses were also applied to internodal strands at frequencies of 5–20 Hz, train duration 1 s, to determine whether the impaled cells received slow synaptic input. Tetrodotoxin (TTX), biocytin and CsCl were obtained from Sigma.

Summarised electrophysiological data are presented as mean ± S.D. Student's *t*-test (paired data, one-tailed) was used to assess differences between control and experimental data sets. Unpaired data were compared using a one-way analysis of variance with a post hoc Tukey–Kramer multiple comparisons test. Differences were considered statistically significant at $P < 0.05$.

After recording, neurons were injected with biocytin using the protocol of Messenger *et al.*[36] Once a neuron in a ganglion had been injected with biocytin, the ganglion was drawn and the recording electrode was moved to a fresh ganglion to avoid ambiguity of cell identity. At the end of each experiment, the tissue was fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0), cleared in three changes of dimethylsulphoxide and placed in phosphate-buffered saline. The tissue was then reacted with streptavidin coupled to Texas Red to reveal biocytin. To analyse the morphologies and projections of the impaled neurons, preparations in which impaled nerve cells had been identified were removed from the slides and washed in phosphate-buffered saline, prior to conversion of the streptavidin, bound to the biocytin, to a permanent deposit. This was achieved using goat anti-streptavidin antiserum coupled to biotin (Vector Laboratories, Burlingame, CA, USA), diluted 1:50 at room temperature. The biotin was in turn localised using an avidin–biotin–horseradish peroxidase kit (Vectastain, Vector Laboratories). The horseradish peroxidase was reacted with diaminobenzidine and hydrogen peroxide to yield a permanent deposit.

Cell shapes, positions and projections were evaluated on an

Table 1. Characteristics of the types of neuron encountered in the submucosal plexus of the guinea-pig distal colon

	S neurons	AH neurons	LT neurons
Resting membrane potential (mV)	− 49 ± 5	− 59 ± 8*	− 51 ± 7
R_{in} (MΩ)	290 ± 120	260 ± 110	430 ± 190*
Action potential threshold (pA)	110 ± 75	85 ± 30	30 ± 20†
Action potential half-width (ms)	1.4 ± 0.2*	2.2 ± 0.4*	1.8 ± 0.5*
Shoulder on action potential	No	Yes	No
Fast EPSPs	Yes	No	No
Slow EPSPs	Yes	Yes	Yes
I_H	No	Yes	Yes
I_A	Yes	No	No
TTX-insensitive action potential	No	Yes	Yes
Late AHPs	No	Yes	No
Morphology	Uniaxonal	Dogiel type II	Uniaxonal

Data are mean ± S.D.
*Significantly different to the other two types.
†Significantly different to S neurons.

Olympus BH microscope under positive-low phase contrast optics, and drawn with the aid of a camera lucida drawing tube at ×400 or ×1000 magnification.

RESULTS

Intracellular recordings were made from 110 neurons within the submucosal ganglia of the guinea-pig distal colon, 63 of which were identified morphologically after intracellular injection of biocytin. Further electrophysiological analysis was made for 58 of the 63 neurons, all of which were able to generate an action potential in response to a depolarising current pulse. Three types of neuron were identified on the basis of their electrophysiological characteristics (Table 1). One type of neuron had fast EPSPs in response to single stimuli applied to internodal strands; this neuron is referred to as the S type, by analogy with classification schemes used in other gut regions. Another type of neuron was characterised by the presence of a prolonged (duration >4 s) hyperpolarisation following a single action potential in the soma, an inflection on the falling phase of the action potential and a failure to evoke fast EPSPs by internodal strand stimulation. Neurons of this type are referred to as AH. The third type of neuron did not exhibit fast EPSPs, nor did it have late AHP following the action potential. These neurons were very excitable and are referred to as low-threshold (LT) neurons. We recorded from many cells with high resting membrane potentials (−70 to −80 mV) that were inexcitable and were revealed by dye injection to have the morphologies of glial cells.

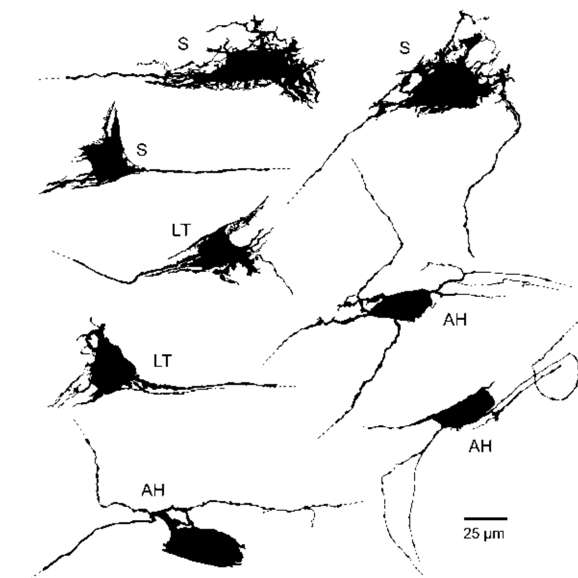

Fig. 1. Camera lucida drawings of the morphologies of neurons that had been characterised electrophysiologically (S, LT or AH, as indicated) and were injected with the intracellular marker biocytin via the recording electrode. Neurons with S or LT electrophysiological characteristics are uniaxonal with a variety of somal morphologies. Many S and LT neurons had very similar shapes, as is evident by comparing the LT neurons illustrated here and the lower of the three S neurons. AH neurons are multiaxonal Dogiel type II neurons. Dotted lines indicate where processes continued.

S neurons

Intracellular recordings identified 33 of 63 neurons (52%) as S neurons. All received fast synaptic input and all S neurons had a single axon (Fig. 1). Twenty-five of 33 S neurons had cell bodies with short lamellar dendrites which Dogiel[12] classified as type I morphology; the remaining eight neurons had long tapering dendrites. Twenty-seven S neurons had axons that left the plane of the plexus close to their cell bodies, while four neurons had anal projections within the plexus, one had an oral projection and another neuron had a circumferential projection. None of these neurons had varicosities within ganglia and thus they appear unlikely to serve as interneurons in the submucosal plexus.

The average resting membrane potential of S neurons was −49 ± 5 mV, the input resistance (R_{in}) was 290 ± 120 MΩ and the threshold for action potential discharge was reached upon intracellular injection of 110 ± 75 pA depolarising current ($n = 33$ neurons for each measurement; Table 1). Nineteen S neurons displayed slow accommodation of action potential firing, discharging 29 ± 6 action potentials during 500-ms depolarising pulses. The other 14 neurons were rapidly accommodating, firing 6 ± 4 action potentials. The

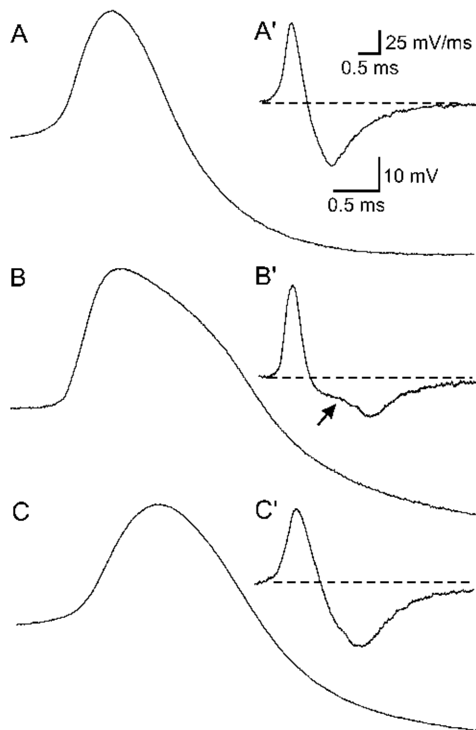

Fig. 2. Action potential characteristics of the three types of neuron defined in the present study. (A) S neurons had relatively brief action potentials with a monophasic repolarisation, as indicated by the lack of inflection in the first time derivative of the voltage trace (A′). (B) An action potential in an AH neuron. The action potential in this neuron, as in all AH neurons encountered, had a prominent shoulder on the repolarising phase of its action potential, which is further revealed by an inflection (arrow) in the first time derivative (B′). (C) LT neurons had action potentials that were broader than those of S neurons, but did not have a noticeable shoulder on the repolarising phase. This is shown by the lack of an inflection in the first time derivative (C′). Calibration for A applies to B and C; calibration for A′ applies to B′ and C′.

average width of action potentials at half-amplitude for S neurons was 1.4 ± 0.2 ms and there was no shoulder on the repolarising phase (Fig. 2A). Twenty-four S cells appeared to have an A-type potassium conductance (I_A). This conductance was evident as a delay in the return to rest of the membrane potential following release of a hyperpolarising current pulse (Fig. 3A) and it appeared at an apparent membrane potential of −68 ± 5 mV. There was no correlation between cells that had I_A and cells that displayed rapid or slow accommodation to action potential firing. TTX (300 nM), which blocks the majority of voltage-gated sodium channels in vertebrate neurons, reversibly abolished action potential firing in each of five S neurons examined, regardless of the stimulus intensity used.

Fast EPSPs were evoked in all S neurons by single stimuli applied to internodal strands (Fig. 4A). Fast EPSPs were recorded in the absence of any stimulus in 15 of 33 neurons (Fig. 4A′). The amplitude of evoked fast EPSPs was increased by increasing the stimulus intensity or by hyperpolarising the membrane potential, and often reached the threshold for action potential firing. The average amplitude of subthreshold fast EPSPs evoked at resting membrane potential was 13 ± 3 mV ($n = 16$). The effect of the nicotinic receptor antagonist hexamethonium (100 μM) on fast EPSPs was tested in 12 neurons. In 10 neurons, fast EPSPs were abolished (Fig. 4A), and in the other two fast EPSPs in the presence of hexamethonium were 50% and 90% of control values. High-frequency (5–20 Hz) presynaptic stimuli evoked slow EPSPs in 18 of 25 (72%) S neurons examined. Slow EPSPs had amplitudes that ranged from 3 to 15 mV at resting membrane potential, and durations of between 20 and 60 s. On no occasions were inhibitory postsynaptic potentials evoked.

AH neurons

AH neurons were characterised by a late AHP following a single soma action potential. Late AHPs were observed in eight of 63 neurons (13%) whose cell bodies were revealed by biocytin injection. Each AH neuron had a smooth cell body with several long axonal processes (Fig. 1), originally defined as type II by Dogiel.[12] The axons of these neurons provided dense innervation of between three and eight adjacent submucosal ganglia (Fig. 5), and are also known to project to the mucosa.[34] Five of 63 neurons (8%) that were inexcitable for the duration of an impalement, defined as type 3 neurons by Frieling *et al.*,[15] also displayed Dogiel type II morphology.

The average resting membrane potential of AH neurons was −60 ± 8 mV, R_{in} was 260 ± 110 MΩ and the threshold for action potential firing was reached following injection of 85 ± 30 pA depolarising current ($n = 8$; Table 1). Upon depolarisation by intracellular injection of current (500 ms), these cells fired up to a maximum of 7 ± 5 action potentials. Action potentials of AH neurons had widths at half-amplitude of 2.2 ± 0.3 ms. The repolarising phase of the action potentials was characterised by a shoulder (Fig. 2B), presumably caused by the influx of calcium ions through voltage-gated calcium channels.[22] This shoulder was further illustrated by examination of the first time derivative of the action potential (Fig. 2B′). When single action potentials were evoked in AH cells, a short (duration ~60 ms) and a long-lasting (duration >4 s) hyperpolarisation followed (Fig. 4B′). Single electrical stimuli applied to internodal strands evoked antidromic action potentials in five of six AH neurons, but never evoked fast EPSPs. Slow EPSPs were evoked by trains of stimuli applied to internodal strands in four of six AH neurons, the amplitudes and durations of which were 3–4 mV and 20–50 s, respectively.

The effect of TTX on action potential firing in four AH neurons was examined. TTX (300 nM) abolished antidromic action potentials in each cell and significantly reduced the maximum number of action potentials that were evoked by intracellular injection of depolarising current from 10 ± 5 to 3 ± 2 ($P < 0.05$; $n = 3$); the fourth AH neuron fired a single action potential before and after the addition of TTX. TTX also significantly increased the threshold amount of depolarising current required for action potential discharge from 75 ± 50 to 170 ± 100 pA ($P < 0.05$; $n = 4$). The effect of lowering the concentration of extracellular Ca^{2+} from 2.5 to

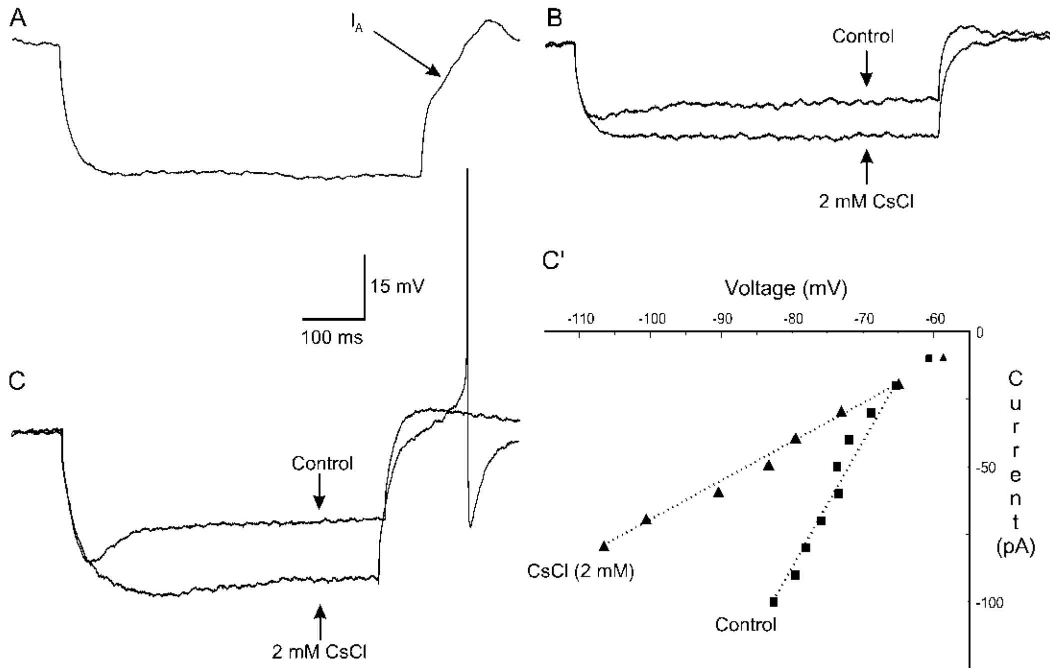

Fig. 3. Response of the membrane potential to hyperpolarisation by 500-ms rectangular current pulses. (A) In many S neurons, a delay in repolarisation following the release of a hyperpolarising current pulse (80 pA) suggests that there is an I_A current in these neurons (at the arrow). (B) In AH neurons, a relaxation of the hyperpolarisation of the membrane potential to injection of a 40-pA current pulse is observed that is blocked by CsCl (2 mM), indicating the expression in AH neurons of I_H. (C) I_H is also seen in all LT neurons (hyperpolarising current pulse of 60 pA used). Calibration applies to A–C. (C′) Comparison of the steady-state voltage response of the LT neuron membrane to increasing amplitudes of hyperpolarising current pulses before and after the addition of CsCl. Measurements were taken 400 ms after the start of the current pulse (arrows in C).

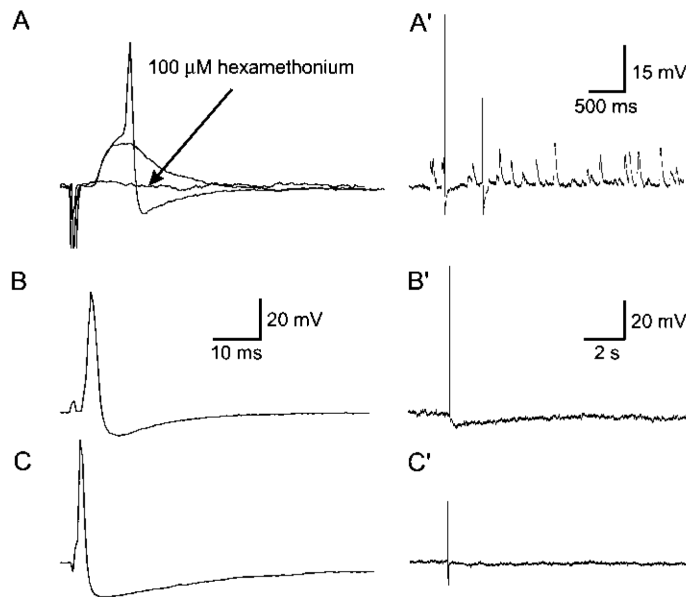

Fig. 4. Responses of S, AH and LT neurons to single stimuli applied to internodal strands. (A) Stimulation of internodal strands results in fast EPSPs in S neurons. Increasing stimulus intensity results in action potential threshold being reached. Fast EPSPs in most S neurons were abolished by 100 µM hexamethonium. (A′) An example of spontaneous fast EPSPs recorded in an S cell. Two of the fast EPSPs are suprathreshold. (B, C) Internodal strand stimulation evokes antidromic action potentials in both AH and LT neurons, but not fast EPSPs. (B′) A single action potential is followed by a late AHP in an AH neuron. (C′) A single action potential does not evoke a late AHP in an LT neuron. The calibration on the left applies to A–C. The top right-hand calibration applies to A′ and the lower right-hand calibration applies to B′ and C′.

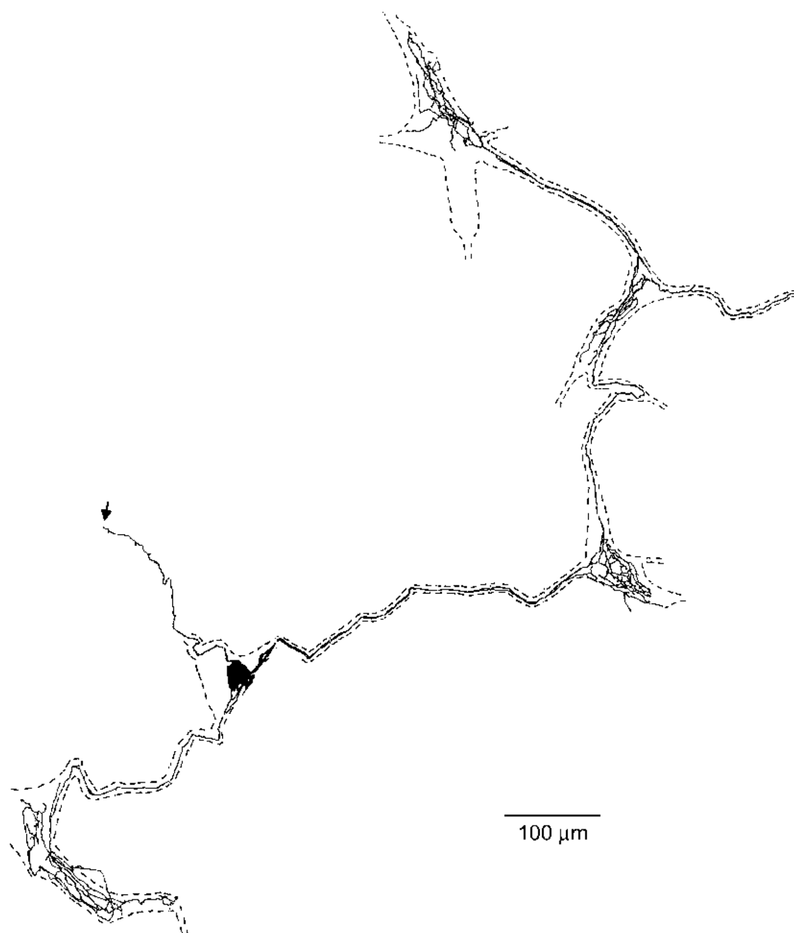

Fig. 5. Innervation of the submucosal ganglia by the axons of an AH neuron. A camera lucida drawing of an AH neuron that had been injected with biocytin through the recording electrode. The neuron has three axons, two of which provide varicose terminals in the submucosal ganglia, the other leaving the plane of the plexus (arrow). Dotted lines outline ganglia and internodal strands.

0.25 mM and increasing the concentration of Mg^{2+} from 1.2 to 10 mM was examined in two AH neurons. Superfusion of preparations with low Ca^{2+}, high Mg^{2+}-containing saline reduced the maximum number of action potentials in these cells from 9 to 5 and from 7 to 2. Surprisingly, in two cases where preparations were superfused with TTX (300 nM) in low-calcium-containing saline, AH neurons were still able to fire action potentials in response to depolarising current pulses.

Six of eight AH neurons had a time-dependent relaxation of the hyperpolarising response of the membrane potential to intracellular current injection (Fig. 3B). The relaxation occurred when neurons were hyperpolarised to potentials more negative than -81 ± 2 mV ($n = 6$) and was increased in amplitude with larger hyperpolarisations. This relaxation was abolished by CsCl (2 mM; Fig. 3B) in three cells tested, implying that it was due to the activation of hyperpolarisation-activated cation current (I_H). CsCl depolarised the resting membrane potential by 5 ± 3 mV ($n = 3$). The amplitude of the late AHP that followed single action potentials was increased following addition of CsCl to the superfusion solution, as reported by Galligan *et al.*[20] for AH neurons in the myenteric plexus of the guinea-pig small intestine.

Low-threshold neurons

Neurons that had certain properties in common with AH neurons were impaled. These included 17 of 63 neurons (27%) that were well filled with biocytin. Fast EPSPs were never recorded in response to internodal strand stimulation and each of these neurons had a relaxation in the response of the membrane potential to a maintained hyperpolarisation. However, their action potentials did not have shoulders on their falling phases (Fig. 2C, C′), and single action potentials were never followed by late AHPs (Fig. 4C′, Table 1). All of these neurons were uniaxonal, with 13 having Dogiel type I morphology and the other four having long tapering dendrites. The axons of each of these neurons projected very short distances before leaving the plane of the plexus, ending in an expansion bulb which results from the accumulation of axoplasm following severing of an axon during dissection.

LT neurons had very high input resistances (430 ± 190 MΩ; $n = 17$) and low thresholds for action potential firing, requiring only 30 ± 20 pA ($n = 17$) of depolarising current to evoke regenerative potentials. The average resting membrane potential of LT neurons was -51 ± 7 mV ($n = 17$). Slow accommodation was

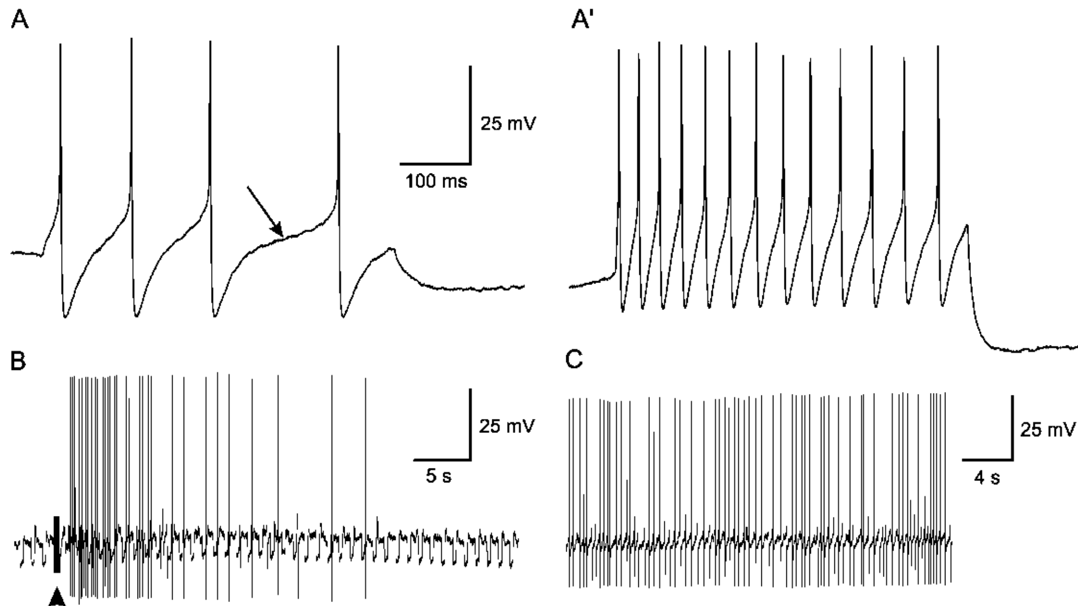

Fig. 6. Electrophysiological characteristics of LT neurons. (A, A′) Action potential discharge in the same neuron in response to injection of 20-pA (A) and 100-pA (A′) depolarising current pulses. In A, the LT neuron fires action potentials for the duration of the imposed depolarisation, although at low frequency, whereas in A′ the larger depolarising current pulse gives a greater frequency of discharge. In A, the repolarisation of the early AHP consists of a fast and a slow component; the fast component is probably due to inactivation of a delayed rectifier potassium conductance, but the identity of the conductance change underlying the slow component (arrow) is unclear, although CsCl (2 mM) does not affect it. (B) An example of a slow EPSP caused by 10-Hz electrical stimulation of an internodal strand for 1 s (arrowhead). Note the dramatic increase in excitability following the stimulus, despite the modest membrane depolarisation, suggesting that the resting membrane potential in this neuron is very close to the threshold for action potential discharge. Downward deflections are voltage responses to applied current pulses, used to monitor input resistance. (C) An example of spontaneous action potential discharge in an LT neuron. The instantaneous spike frequency is 3 ± 1 Hz.

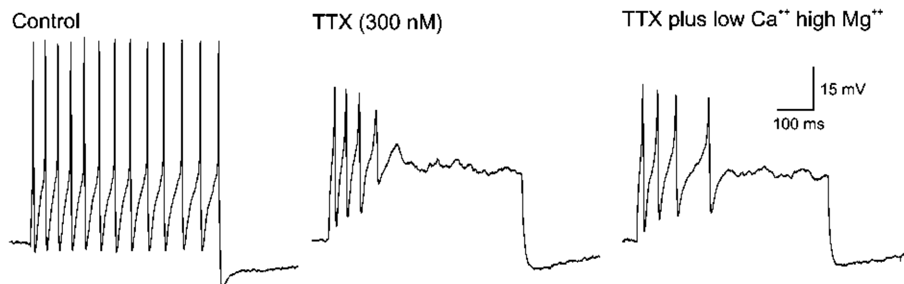

Fig. 7. Effects of blockade of voltage-gated Na^+ and Ca^{2+} conductances on regenerative potentials in a single LT neuron. The amplitude of depolarising current used in each case is 100 pA. TTX (300 nM) reduces the amplitude and the duration of discharge of action potentials in LT neurons. Superfusion of the preparation with low Ca^{2+}, high Mg^{2+} saline containing 300 nM TTX does not appear to further affect action potential discharge, suggesting that voltage-gated Ca^{2+} conductances are not involved in action potential generation in LT neurons.

observed in each of these neurons, and increasing the intensity of depolarising current increased the number of action potentials discharged during a 500-ms current pulse (Fig. 6A, A′) to a maximum of 15 ± 6 action potentials ($n = 17$). The width of the action potential of LT neurons at half-amplitude was 1.8 ± 0.5 ms ($n = 17$), which is longer than that of S neurons, but there were never discernible shoulders on the repolarising phases of action potentials of LT neurons (Fig. 2C). LT neurons often discharged spontaneous action potentials during an impalement; an example is shown in Fig. 6C. The instantaneous frequency of action potential firing in this example is 3 ± 1 Hz. Antidromic action potentials were evoked in 12 of 17 neurons in response to internodal strand stimulation (Fig. 4C). High-frequency stimuli applied to internodal strands evoked slow EPSPs in three of eight LT neurons (Fig. 6B). Slow EPSPs had amplitudes of between 5 and 8 mV, and durations ranging from 20 to 30 s.

TTX (300 nM) significantly reduced the number of action potentials that could be elicited by 500-ms depolarising current pulses from 14 ± 1 to 3 ± 5 ($P < 0.05$; $n = 5$; see Fig. 7). TTX also significantly increased the threshold amount of depolarising current needed to evoke action potentials in LT neurons from 20 ± 10 to 70 ± 50 pA ($P < 0.05$; $n = 5$). Antidromic conduction of action potentials to the cell bodies was blocked by 300 nM TTX ($n = 5$). Action potential firing in LT

neurons was unaffected by lowering the extracellular Ca^{2+} concentration (Fig. 7), although superfusion of preparations in low-Ca^{2+}-containing saline depolarised the membrane potential by 4 ± 2 mV ($n = 5$), which suggests the presence of a calcium-activated potassium conductance that is open at rest. Application of CsCl significantly reduced the maximum number of action potentials that could be evoked during a 500-ms depolarising pulse from 13 ± 2 to 10 ± 3 action potentials ($P < 0.05$; $n = 5$). The repolarisation of the membrane following the action potential had two phases (Fig. 6A). The first phase was rapid and was probably due to the inactivation of a delayed rectifier potassium current. The current underlying the second phase (arrow in Fig. 6A) is unknown, but it was not sensitive to CsCl (2 mM).

Following the end of a large-amplitude (>50 pA) depolarising current pulse applied to LT neurons, there was often a hyperpolarisation of the membrane potential (e.g. Figs 6 and 7). The amplitude of this hyperpolarisation was not related to the number of action potentials elicited by the prior depolarising current injection.

A characteristic feature of LT neurons was a relaxation of the hyperpolarising response of the membrane potential to current injection. This relaxation was noticeable when the membrane potential was stepped from resting values to -73 ± 10 mV ($n = 17$) or greater hyperpolarisation, with the voltage relaxation increasing in size with larger hyperpolarisations. Superfusion of preparations with CsCl (2 mM) caused a depolarisation of the membrane potential, in three of five neurons tested, by 7 ± 2 mV. In all five neurons, CsCl caused a reversible block of the voltage relaxation, suggesting that it is due to the expression in these cells of I_H.[20] Superfusion with CsCl did not reveal a late AHP in LT neurons.

DISCUSSION

The present study has identified three types of neuron that reside within the submucosal ganglia of the guinea-pig distal colon. Distinctions between neurons were based on differences in their membrane responses to current injection, differences in the nature of synaptic inputs that they receive, and differences in morphologies and projections (Table 1). Two of the types of submucosal neuron (S and AH) have been documented in other regions of the gastrointestinal tract of guinea-pigs, while the third type (LT neurons), with the exception of a study by Surprenant[42] in the guinea-pig small intestine, does not appear to have been encountered before. A previous electrophysiological study of submucosal neurons in the colon identified S (type 1) and AH (type 2) neurons, as well as neurons that were inexcitable (type 3) or had low excitability (type 4).[15]

S neurons

Neurons with S-type electrophysiological characteristics comprised the largest population of neurons within the submucosal ganglia of the distal colon. Fast EPSPs were evoked by internodal strand stimulation in all S neurons. Action potential discharge was sensitive to TTX, as is the case for S neurons in all regions of the gastrointestinal tract examined.[5,44] Fast EPSPs evoked by internodal strand stimulation were abolished by hexamethonium in 10 of 12 neurons. The non-cholinergic transmitter of fast EPSPs was not identified. One candidate transmitter is ATP acting at P_{2X} receptors.[1,32] There is also evidence that serotonin and/or glutamate may play a role in fast excitatory transmission.[33,46]

All S neurons had uniaxonal morphologies, which agrees with the observations of Evans *et al.*,[14] who examined the shapes and projections of S neurons in the submucosal plexus of the small intestine. They reported that the axons of S neurons often projected to other submucosal ganglia and had varicose regions and tufts of varicosities within these ganglia. In the present work, six of the S neurons had long axonal projections within the submucosal plexus; four of these had oral to anal polarity. Although we cannot exclude the possibility, we found no evidence of varicose regions of axons of S neurons within the submucosal ganglia. The presence of expansion bulbs suggests that the axons of S neurons leave the plane of the plexus, and recent immunohistochemical data suggest that uniaxonal submucosal neurons that project to the mucosa in the distal colon are secretomotor neurons.[34]

AH neurons

We have identified a class of neuron with the same electrophysiological and morphological characteristics as IPANs in the ileum.[3,19,21,29,30,42] These neurons had AH electrophysiological characteristics, lacked fast synaptic input and had multiaxonal Dogiel type II morphology. Bornstein *et al.*[3] were able to record fast EPSPs in a minority of AH neurons (all of which were immunoreactive for substance P) in the submucosal plexus of the ileum, although fast EPSPs were of lower amplitude than those recorded in S cells in the same preparation. Surprenant[42] reported that AH neurons in these ganglia lacked fast EPSPs. In the submucosal ganglia of the transverse colon, Frieling *et al.*[16] reported that 70% of type II neurons had fast EPSPs. One difference between the present study and that of Frieling *et al.*[15] is that Frieling *et al.* used colon 10–20 cm distal to the colonic flexure, whereas we used segments 2–5 mm from the pelvic brim (35–40 cm distal to the flexure). Cunningham and Lees[11] examined different regions of the guinea-pig intestine using immunohistochemical techniques, and reported that the population of submucosal neurons in the region used by Frieling *et al.*[15] (called the transverse colon by Cunningham and Lees) is significantly different from that of the distal colon (called the descending colon by Cunningham and Lees) that we examined. For example, the proportion of neuropeptide Y-immunoreactive neurons is three times greater in the transverse colon, compared to the descending colon.

The targets of the axons of Dogiel type II neurons with cell bodies in the submucosal ganglia have been a subject of debate. The neurons project to the myenteric plexus and to the mucosa,[28,38,41] and perhaps to other submucosal ganglia, although evidence in favour of this

last projection was not strong prior to this study. Immunohistochemical studies suggest that all substance P-immunoreactive terminals in the submucosal ganglia of the small intestine originate from neurons with cell bodies extrinsic to the gut or in the myenteric plexus.[10,27] Thus, it was thought that AH neurons in the submucosal plexus, whose cell bodies are all substance P immunoreactive, did not innervate other submucosal neurons. Evans *et al.*[14] found that neurons with AH electrophysiological characteristics projected to other submucosal ganglia in the small intestine. However, the innervation of other submucosal ganglia by submucosal Dogiel type II neurons was found to be much sparser than that observed for the innervation of myenteric ganglia by the axon terminals of myenteric Dogiel type II neurons.[6,14] We have found that neurons with AH electrophysiological characteristics provide dense innervation to other submucosal ganglia, comparable to that seen in the myenteric plexus, which suggests that Dogiel type II neurons in the submucosal plexus of the distal colon can innervate a large number of other submucosal neurons. Given that submucosal Dogiel type II neurons also project axons to the mucosa, it is likely that these neurons can detect mucosal stimuli, as suggested by Kirchgessner *et al.*,[29,30] and directly innervate secretomotor and vasomotor neurons in the submucosal plexus, thus providing the neural circuitry for a monosynaptic secretomotor reflex, which has been suggested to exist in the colon.[40]

Low-threshold neurons

The present work has identified a type of enteric neuron, LT neurons, that have properties that set them apart from those encountered in almost all investigations of enteric neurophysiology to date. S neurons, by definition, receive fast synaptic input, and hence LT neurons cannot be classed as S neurons because fast EPSPs were never recorded. Moreover, soma action potentials in S neurons are fully abolished by TTX. In the myenteric plexus, AH neurons often lack the late AHP that was originally their defining characteristic.[5,44] This is because a background level of slow excitatory transmission to these neurons can inhibit the calcium-activated potassium conductance that underlies the AHP in Dogiel type II neurons (see Ref. 19). Initially, we thought that LT neurons might be AH/Dogiel type II neurons that are under the influence of slow EPSPs. Two observations refuted this possibility. First, Dogiel type II neurons have a calcium component of their action potentials, seen as a shoulder on the falling phase, whereas LT neurons had no calcium component and no shoulder. Second, LT neurons had uniaxonal morphologies, which also distinguishes them from AH neurons, which have Dogiel type II morphology.

Surprenant[42] described "spontaneously active" neurons in the submucosal ganglia of the small intestine. Spontaneously active neurons, as the name implies, often generated bursts of action potentials. We have observed spontaneous action potential discharge in some LT neurons. Spontaneously active neurons in the ileum had action potentials that were resistant to TTX and to reduction of extracellular calcium concentration, a trait shared by the LT neurons of this study. One characteristic of LT neurons differs from those of the spontaneously active neurons. Surprenant[42] reported that spontaneously active neurons never received slow excitatory synaptic input, whereas LT neurons often had slow EPSPs in response to internodal stand stimulation. This discrepancy may have been due to differences in the method of stimulation used. Surprenant[42] used transmural stimulation at a distance of up to 4 mm, which may not have activated all the fibres impinging on LT neurons as effectively as the focal stimulation used here.

On the basis of their uniaxonal morphologies, it is likely that LT neurons function as secretomotor neurons. Sidhu and Cooke[40] recorded a TTX-sensitive submucosal secretory reflex in the distal colon that was insensitive to blockade of nicotinic acetylcholine receptors. As LT neurons receive only slow synaptic input, they might be the output neurons in this secretory reflex.

CONCLUSIONS

The submucosal ganglia of the distal colon contained three electrophysiologically defined neuronal types, which have also been identified by shape and, from previous data, can be tentatively functionally identified. Neurons identified electrophysiologically as the S type received fast and slow EPSPs and had soma action potentials that were blocked by TTX. These were uniaxonal and are deduced to be secretomotor or secretomotor/vasodilator neurons. Neurons identified electrophysiologically as the AH type received only slow EPSPs and had action potentials with an inflection on the falling phase that were followed by prolonged AHPs. Many had a hyperpolarisation-activated current; soma action potentials were reduced but not blocked by TTX. These neurons were multipolar, Dogiel type II neurons. AH/Dogiel type II neurons are deduced to be intrinsic primary afferent neurons. Neurons that were identified electrophysiologically as the LT type received only slow EPSPs. All had a prominent hyperpolarisation-activated current, were highly excitable and were sometimes spontaneously active. They were uniaxonal and it is suggested that they are secretomotor neurons.

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