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INTRACELLULAR RECORDING FROM NEURONES OF THE GUINEA-PIG GALL-BLADDER

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SUMMARY

1. Intracellular recordings were made from neurones of the guinea-pig gall-bladder *in vitro*. Intracellular injection of horseradish peroxidase revealed a simple structure, consisting of a soma and a single process, but no discernible dendritic arborization.

2. The resting membrane potential was $-50{\cdot}5 \pm 0{\cdot}4$ mV and the input resistance was 80 MΩ.

3. Gall-bladder neurones spiked only once at the onset of depolarizing current pulses. Action potentials were blocked by tetrodotoxin, but a Ca^{2+}-dependent spike could be elicited in the presence of tetrodotoxin and tetraethylammonium.

4. Action potential after-hyperpolarizations had a duration of $172 \pm 3{\cdot}7$ ms and reversed at a membrane potential of -93 mV; this reversal potential was linearly related to the logarithm of the external potassium concentration. The initial phase of the after-hyperpolarization was inhibited by tetraethylammonium (1–10 mM) and was not affected by 3,4-diaminopyridine. The late phase of the after-hyperpolarization was blocked by apamin (10 nM) or curare (500 μM). Both the early and late phases of the after-hyperpolarization were inhibited when the preparation was perfused with a calcium-free, high-magnesium solution. The calcium-free, high-magnesium solution had no effect on the membrane potential or input resistance of these cells.

5. Fast excitatory synaptic responses and antidromic responses were elicited in gall-bladder neurones by focal stimulation of fibre tracts. High-frequency fibre tract stimulation often resulted in prolonged, calcium-dependent, depolarizations that were associated with a decrease in input resistance.

6. 5-Hydroxytryptamine and substance P caused depolarizations that were associated with a decrease in input resistance. Bethanechol caused hyperpolarizations that were associated with a decrease in input resistance and which were blocked by atropine.

INTRODUCTION

Circulating hormones are known to play a significant physiological role in the regulation of the motor and absorptive functions of the gall-bladder (see Ryan,

1987). In addition, well-defined neural plexuses are present in the wall of the organ, but the physiological purpose of this innervation, relative to the hormonal control, is not well understood. The presence of intrinsic neurones, as well as plexuses of terminal axons in proximity to the epithelium and smooth muscle, suggest that the nervous system may play a role in governing the activity of the gall-bladder tissues. In view of the gall-bladder's origin as an outgrowth of the fetal gut, it is reasonable to expect that the ganglionated neural plexus of the gall-bladder is analogous to one or both of the plexuses of the enteric nervous system and, like the enteric nervous system, that it contains secretomotor neurones and/or neurones that excite or inhibit smooth muscle.

Recent studies indicate that the ganglionated plexus of the gall-bladder is similar – in structure, diversity of transmitter content and connections – to the ganglionated plexuses of the small intestine (see Cai & Gabella, 1983; Mawe & Gershon, 1989). In common with enteric ganglia, but unlike other peripheral ganglia, laminin immunoreactivity is excluded from the interior of gall-bladder ganglia, which are surrounded by a periganglionic laminin-immunoreactive sheath. The ganglionated plexus of the gall-bladder contains neurones and/or fibres that are immunoreactive for 5-hydroxytryptamine (5-HT), tyrosine hydroxylase, substance P, calcitonin gene-related peptide, neuropeptide Y, somatostatin, enkephalin and vasoactive intestinal polypeptide. The extrinsic innervation of the gall-bladder is derived from the dorsal motor nucleus of the vagus (parasympathetic), the coeliac (sympathetic), nodose (sensory) and thoracic dorsal root ganglia (T5–T11). In addition, neurones in ganglia of the myenteric plexus of the duodenum, near the sphincter of Oddi, project to the gall-bladder.

The present study was undertaken to determine whether the electrophysiological characteristics of guinea-pig gall-bladder neurones are analogous to those of small intestine neurones, as well as to ascertain the nature of synaptic input to gall-bladder neurones. To evaluate the physiological similarities (or dissimilarities) between the ganglia of the gall-bladder and those of the bowel, properties known to be characteristic of enteric neurones received special attention. These properties include repetitive spike discharge and anodal break excitation which are characteristic of type I/S cells of the small intestine, and tetrodotoxin-insensitive spikes and prolonged after-hyperpolarizations (AHPs) which are characteristic of type II/AH cells (see Wood, 1987).

METHODS

Guinea-pigs (200–350 g) were stunned by a blow to the head and then exsanguinated. The gall-bladder and cystic duct were quickly removed, washed with Krebs solution, and cut open with a single incision from the end of the cystic duct to the base of the gall-bladder. The tissue was then pinned flat, mucosal side up, under recirculating iced Krebs solution in a dish lined with Sylgard 184 elastomer (Dow Corning Corp, Midland, MI, USA). The mucosal layer and much of the smooth muscle of the gall-bladder were gently removed with forceps under microscopic observation. The preparations were then transferred to a tissue chamber and placed on the stage of an inverted microscope (Nikon, Diaphot). Individual ganglia were visualized at $\times 200$ with Hoffman modulation contrast optics.

For physiological recording, the preparations were continuously perfused at 4 ml/min with a modified Krebs solution that contained (mM): NaCl, 121; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 25; NaH_2PO_4, 1·2; and glucose, 8. The solution was gassed with 95% O_2/5% CO_2, and the temperature was 35–37 °C at the recording site.

Glass microelectrodes used for recording were filled with 2 M-KCl and had resistances in the range of 60–90 MΩ. Synaptic and antidromic activation of neurones was elicited by direct stimuli (1–10 V; 200–250 μs duration, 0·5–20 Hz frequency) applied to fibre tracts in interganglionic connectives with monopolar extracellular electrodes made from Teflon-insulated platinum wire (25 μm diameter). All results were recorded on magnetic tape for subsequent analysis.

Drugs were applied by replacing the normal Krebs solution with Krebs solution containing the test substance, or by pressure microejection. When pressure microejection was used, drugs (0·1–1 mM) were applied from micropipettes (15–20 μm tip diameter) by pulses of nitrogen gas (300 kg/cm^2; 10–999 ms duration). The distance between the tip of the micropipette and the recording microelectrode was 50–100 μm. In instances when the ionic composition of the circulating solution was altered, the concentration of NaCl was reduced so that the final osmolarity remained constant. All reagents were dissolved directly into Krebs solution. In some experiments, apamin was dissolved at 0·1 mM in dimethyl sulphoxide and further diluted in Krebs buffer. Results from these experiments were not different from those with direct dilution in Krebs buffer. Tetrodotoxin (TTX), tetraethylammonium (TEA), hexamethonium, *d*-tubocurarine chloride, apamin, 5-HT, substance P and bethanecol were purchased from Sigma Chemical Company (St Louis, MO, USA).

Acetylcholinesterase staining. The methods to demonstrate acetylcholinesterase activity in the gall-bladder have been described in detail in a recent publication (Mawe & Gershon, 1989). Briefly, tissues were fixed for 2 h at room temperature with 4% paraformaldehyde in 0·1 M-phosphate buffer, and then incubated overnight in a medium consisting of 25 mg acetylthiocholine iodide dissolved in 10 ml of each of the following: sodium acetate (0·02 M), acetic acid (0·01 M), sodium citrate (25 mM), copper sulphate (15 mM), potassium ferrocyanide (2·5 mM). Tetraisopropyl-pyrophosphoramide (iso-OMPA; 80 μM) was added to the solution to inhibit butyrlcholinesterase activity. Incubation was carried out with gentle agitation in a water bath at 37 °C. After several rinses in distilled water, the tissue was mounted on gelatin-coated glass slides, dehydrated in a graded series of ethanols, cleared with xylene, and permanently mounted. The slides were examined using Hoffman modulation contrast and bright-field optics.

HRP intracellular injections. The method used in the present study to inject HRP into single neurones has recently been described in detail (Forehand & Konopka, 1989). Microelectrodes were filled to the shoulder with a 5% solution of HRP in 0·05 M-Tris buffer (containing 1·75% potassium acetate buffer). The shank of the electrode was filled with 2 M-KCl. Depolarizing current pulses (200 ms, 2 nA) were injected at a rate of 2 Hz for 10 min. The tissue was fixed in a solution containing 1·25% glutaraldehyde and 0·5% paraformaldehyde, then thoroughly rinsed with HEPES buffer and processed for the histochemical demonstration of peroxidase using the Hanker–Yates procedure. After the reaction, the tissue was mounted on gelatin-coated glass slides, dehydrated in a graded series of ethanols, cleared with xylene, and permanently mounted. The slides were examined using Hoffman modulation contrast and bright-field optics.

RESULTS

Morphology of individual gall-bladder neurones

Gall-bladder ganglia were similar in appearance and distribution to those of the submucosal plexus (see Mawe & Gershon, 1989). They consisted of one or two neurones located on a single fibre tract, or clusters of cells at the junction of several fibre tracts. Figure 1*A* shows a single ganglion, and associated interganglionic connectives, stained for the presence of acetylcholinesterase. In unstained *in vitro* preparations, as viewed during physiological recording sessions, ganglia appear as triangular or fusiform mounds identifiable by fibre bundles that pass to and from them (Fig. 1*B*). In extensively dissected preparations, individual neurones could be seen within the ganglia; however, it was much more difficult to achieve successful impalements of neurones in these preparations.

Individual neurones of the gall-bladder, stained with intracellular injection of HRP, were very simple in structure. Each gall-bladder neurone consisted of a soma

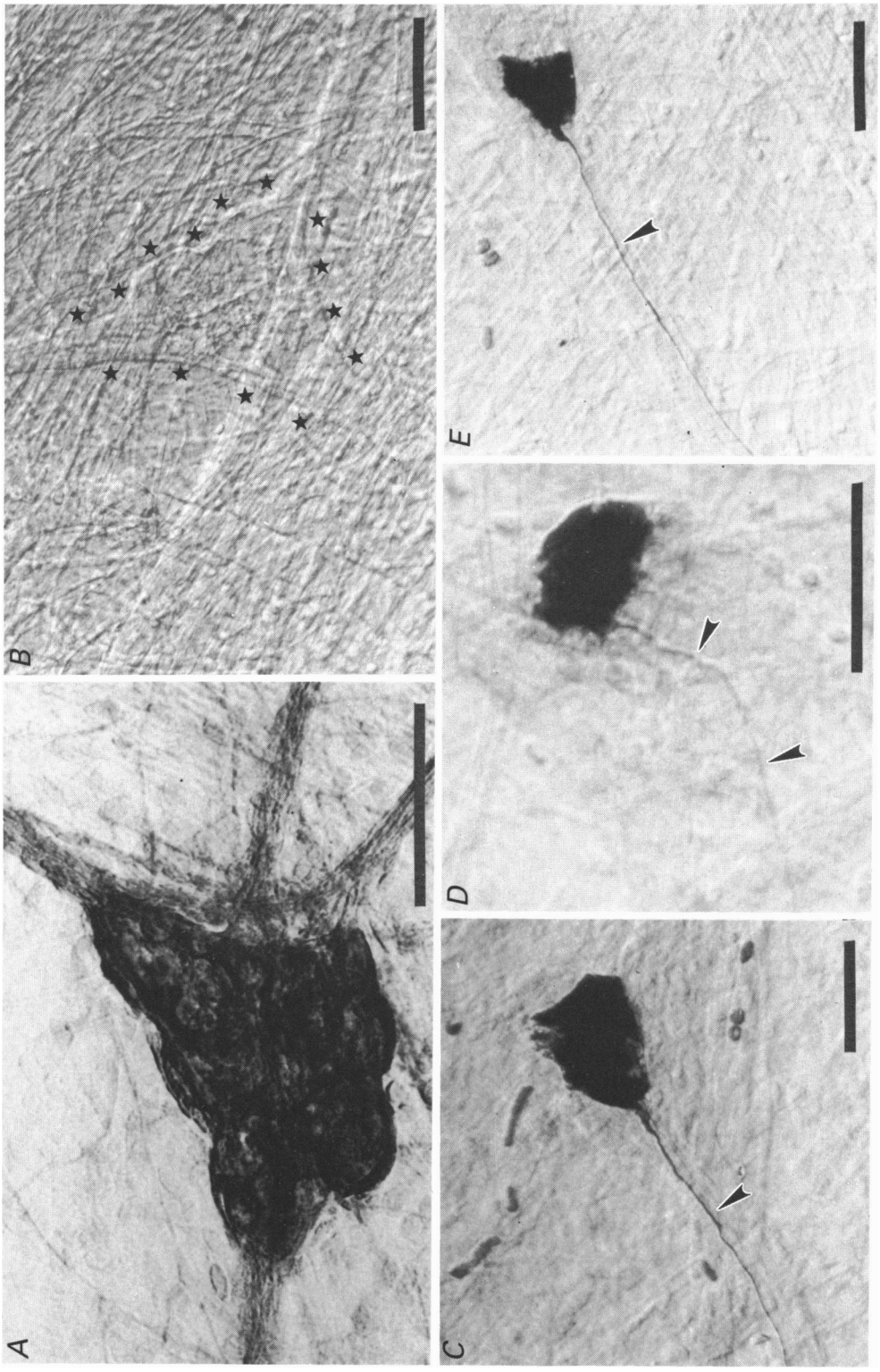

Fig. 1. For legend see facing page.

and a single long process that was thought to be an axon; no dendritic arborization was apparent (Fig. 1*C–E*). The axons usually entered into fibre tracts and could sometimes be traced to other ganglia. Because the mucosa and smooth muscle layers were removed, projections to those putative targets could not be observed. In some neurones, very short protrusions extended from the cell bodies; the nature of these structures will have to be resolved at the ultrastructural level.

Electrical properties of gall-bladder neurones

Intracellular recordings were obtained from 168 cells in which the duration of the impalement (at least 15 min) was sufficient to allow for an initial examination of the electrical properties of a cell and to allow for a determination of whether the cell received fast excitatory synaptic input. This report describes results obtained from 160 cells that had overshooting spikes and responded to fibre tract stimulation.

A single type of gall-bladder neurone was identified on the basis of electrophysiological behaviour. The resting membrane potential was $-50{\cdot}5 \pm 0{\cdot}4$ mV and the input resistance was $79{\cdot}6 \pm 3{\cdot}4$ MΩ ($n = 160$). These values and others in the text are mean ± S.E. of the mean. The time constant, measured as the time elapsed to reach 63% of the steady-state level in response to a 200 ms hyperpolarizing pulse, was $3{\cdot}8 \pm 0{\cdot}2$ ms. The steady-state current–voltage relationship was linear for hyperpolarizing currents, but rectification was observed when the cells were depolarized (Fig. 2*A*). This rectification was considerably reduced when delayed rectifier currents were blocked by the addition of 3,4-diaminopyridine (2 mM) to the bathing solution.

Intracellular injection of a depolarizing current pulse (1–200 ms) usually evoked a single action potential. In some cases two to four spikes occurred if a suprathreshold current pulse was injected, but none of the cells discharged action potentials throughout the duration of a 200 ms depolarizing current pulse. Anodal break excitation was recorded in only 8 of 160 spiking neurones, and in those cases anodal break excitation occurred in response to hyperpolarizing current pulses > 0·2 nA. Typical responses to depolarizing and hyperpolarizing current pulses are seen in Fig. 2.

An additional eight cells that were impaled failed to spike and did not respond to fibre tract stimulation. These non-spiking cells had a resting membrane potential of $-70{\cdot}3 \pm 1{\cdot}7$ mV, and an input resistance of $21{\cdot}0 \pm 2{\cdot}8$ MΩ. Gershon and his colleagues (Erde, Sherman & Gershon, 1985) reported that cells with similar electrophysiological properties in the myenteric plexus of the ileum appear astrocytic in contour, when labelled intracellularly with HRP.

Fig. 1. Structure of gall-bladder ganglia and neurones. *A*, photomicrograph of a gall-bladder ganglion stained for the presence of acetylcholinesterase. *B*, unstained ganglion (surrounded by stars) as seen during physiological experiments. *C*, *D* and *E*, the structural simplicity of individual gall-bladder neurones is seen in these photomicrographs of neurones labelled by intracellular injection of horseradish peroxidase. Axons of these cells are indicated by arrow-heads. Bars = 50 μm.

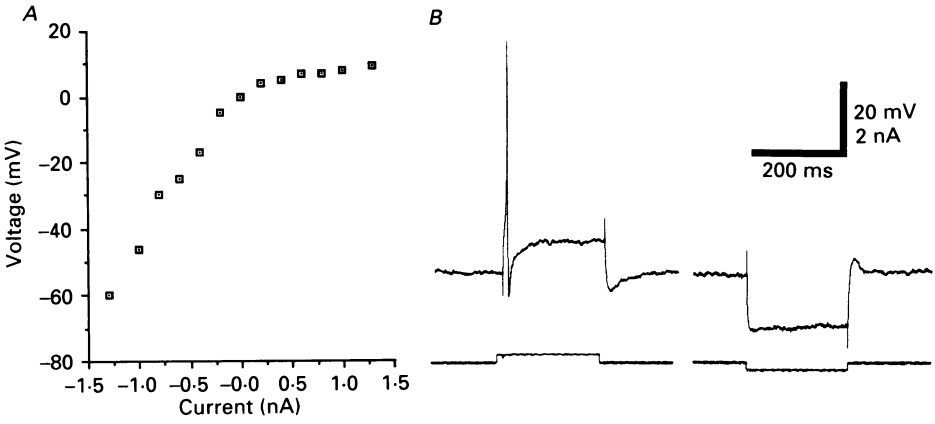

Fig. 2. *A*, steady-state voltage–current relation of a typical gall-bladder neurone. Although a linear relationship is seen in the hyperpolarizing direction, rectification can be seen in the depolarizing direction (control). *B*, neurones of the gall-bladder usually fire a single action potential at the onset of a depolarizing current pulse. Resting potential was −50 mV.

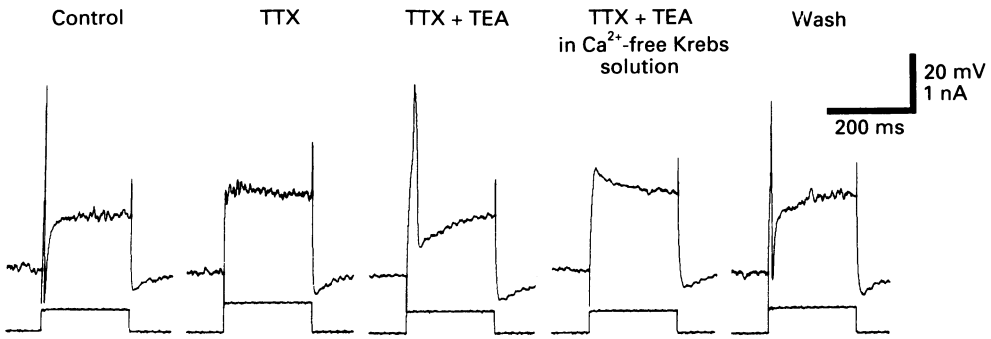

Fig. 3. TTX (0·5 μM) blocks evoked action potentials. Following application of TEA (20 mM) action potentials could be elicited in the presence of TTX. These spikes were eliminated by bathing the cells in a TTX–TEA, calcium-free, high-magnesium solution. Control properties were restored 23 min after rinsing with normal Krebs solution. Resting potential was −49 mV.

Effects of TTX on action potentials

Action potentials were blocked by tetrodotoxin (TTX). Action potentials that were induced by injection of depolarizing current pulses were reversibly blocked by TTX (0·1–0·5 μM) within 30 s in all cells that were tested (Fig. 3; $n = 10$). Increasing the amplitude of depolarizing current pulses did not result in the generation of action potentials in the presence of TTX. The effects of TTX reversed within 20–30 min after wash-out.

In the presence of 20 mM-TEA and TTX, calcium-dependent action potentials could be elicited (Fig. 3). These spikes were broader, and the amplitude of the AHP was less, than those seen in control conditions. The spikes in this solution were abolished when magnesium chloride was raised to 8 mM and calcium chloride was

omitted (Fig. 3). Moreover, when the calcium concentration in the bathing solution was decreased from 2·5 to 1·25 mM the spikes were of smaller amplitude, and when the calcium concentration was increased to 5·0 mM, the spikes were larger in amplitude.

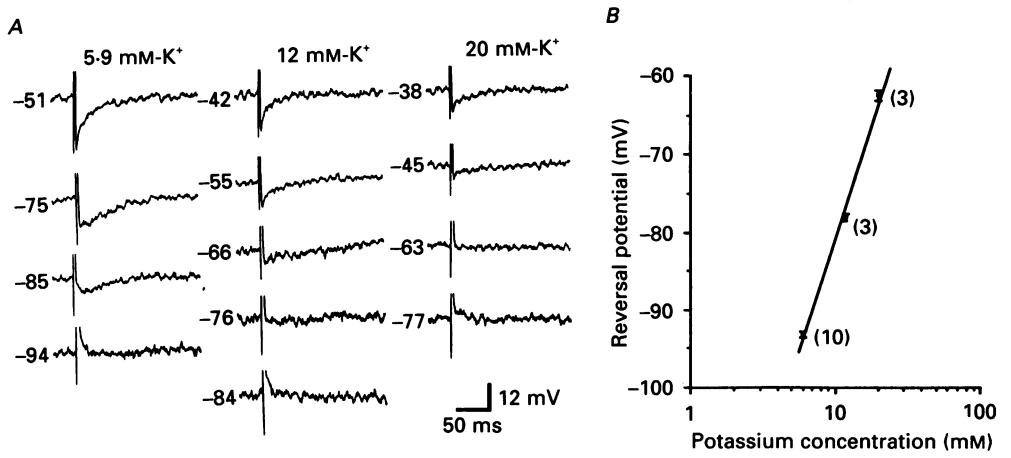

Fig. 4. After-hyperpolarization (AHP) results from potassium conductance increase. *A*, AHPs at different membrane potentials in three different potassium concentrations. *B*, reversal potential in different potassium concentrations. Slope is $-58{\cdot}5$ mV/$\log_{10}$ [K^+]. The number of cells tested at each concentration is indicated in parentheses. Bars are S.E. of mean.

Properties of after-hyperpolarizations

The AHP was $171 \pm 3{\cdot}7$ ms ($n = 66$; range, 123–250 ms) in duration. The amplitude of the AHPs ranged from 9 to 22 mV with a mean of $15{\cdot}3 \pm 0{\cdot}7$ mV ($n = 66$). The amplitude of the AHP increased when the membrane was depolarized and decreased when the membrane was hyperpolarized. Further, the amplitude of the AHP decreased as the potassium concentration of the bathing solution was increased (Fig. 4*A*). The reversal potential of the AHP was shifted by 58·5 mV per decade change in external potassium concentration (Fig. 4*A* and *B*). In control solution, AHP reversal could be observed in only one of ten cells due to an inability to evoke an action potential when the membrane was hyperpolarized beyond -95 mV. However, when the potassium concentration of the bathing solution was increased from 5·9 mM to 12 or 20 mM, the AHP reversed polarity (Fig. 4*A*).

Effects of drugs and calcium-free solution on the after-hyperpolarization

Tetraethylammonium. In the presence of TEA (1 mM), the amplitude of the AHP was decreased to $64 \pm 3{\cdot}1$ % of the control value ($n = 5$; Fig. 5*A*). The duration of the AHPs in these neurones remained the same or was slightly increased. At higher concentrations of TEA (20 mM), the amplitude of AHPs was decreased to $42 \pm 7{\cdot}5$ % of control values ($n = 11$). The higher concentrations of TEA also resulted in a broadening of the action potential with a shoulder appearing during the falling phase of the spike.

3,4-Diaminopyridine. After 10 min in 3,4-diaminopyridine (3,4-DAP; 2 mM), the shape of the action potential and the AHP were very similar to those seen in normal Krebs solution (Fig. 6). When superfusion with 3,4-DAP was followed by superfusion with 3,4-DAP plus TEA (1 mM) there was broadening of the repolarizing phase of the

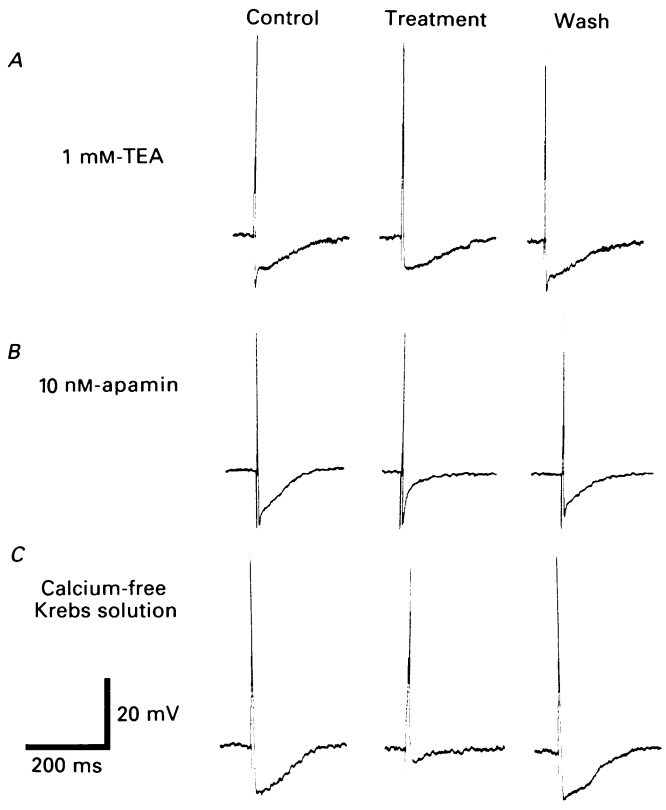

Fig. 5. Effects of TEA, apamin and reduced calcium on the amplitude and duration of the AHP. *A*, TEA decreased the amplitude of the AHP. *B*, apamin (10 nM) decreased the duration but did not affect the amplitude. *C*, the amplitude and duration of the AHP were decreased in the presence of a calcium-free, high-magnesium Krebs solution. Resting potentials were: *A*, −50 mV; *B*, −48 mV; *C*, −52 mV.

action potential (Fig. 6*A*), and there was a decrease in the amplitude of the AHP to 46·5 ± 5·3 % of control values ($n = 4$ cells; Fig. 6*B*). No increase in the number of spikes per depolarizing current pulse (200 ms) was seen in the presence of 3,4-DAP or 3,4-DAP plus TEA.

Apamin and curare. Apamin (10 nM) reversibly decreased the duration of the AHP to 27·1 ± 2·4 % of control durations in nine neurones tested (Fig. 5*B*), but it did not alter the membrane potential or the input resistance of the neurones. Concentrations of apamin ranging from 10 to 100 nM decreased the duration of the AHP without affecting the amplitude. Curare (500 μM) also reversibly decreased the duration of the AHP in all five neurones that were tested. When application of apamin was followed by application of apamin plus TEA, the amplitude of the AHP was reversibly

decreased to 57·8±4·5% of the control value ($n = 5$; Fig. 7). In the presence of apamin or curare, the neurones spiked repetitively throughout a depolarizing current pulse (Fig. 7*A*).

Calcium-free Krebs solution. When control Krebs solution was replaced with Ca^{2+}-free, high-Mg^{2+} Krebs solution, both the amplitude and duration of the AHP were

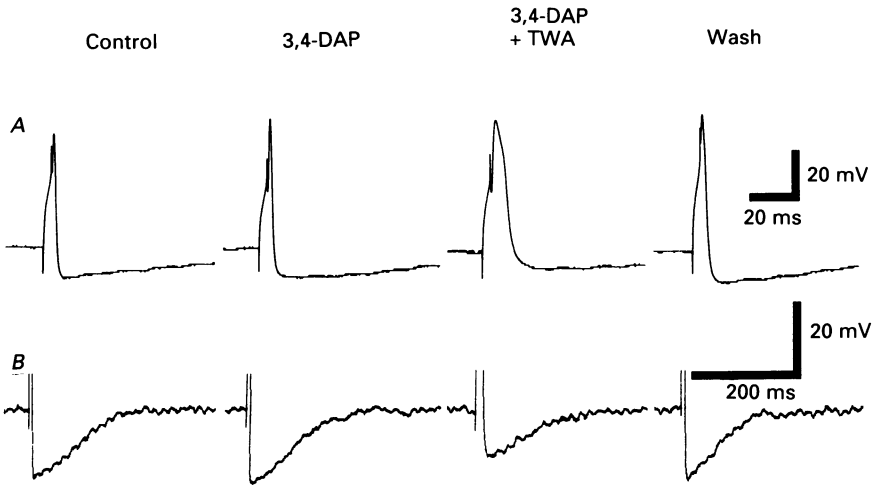

Fig. 6. Effects of 3,4-diaminopyridine (3,4-DAP) and 3,4-DAP plus TEA on the action potential and AHP. 3,4-DAP (2 mM) had no effect on the action potential or AHP. When TEA (1 mM) was added in addition to 3,4-DAP there was a broadening of the spike (*A*) and a decrease in the amplitude of the AHP (*B*). Spikes are truncated in *B*. Resting potential was −49 mV.

decreased (Fig. 5*C*). The amplitude of the AHP was reversibly decreased to 52·4±6·7% of the control in eight neurones tested, and the duration was reversibly decreased to 45·5±5·8% of the control. As in the presence of apamin or curare, the neurones spiked repetitively from the onset of a depolarizing current pulse in Ca^{2+}-substituted, high-Mg^{2+} Krebs solution. Substitution of Mg^{2+} for Ca^{2+} in the superfusion solution did not alter the membrane potential or the input resistance of gall-bladder neurones.

Effect of nerve stimulation

Fast EPSPs

Focal stimulation of fibre tracts with single pulses (0·5 ms duration) evoked fast EPSPs in essentially all of the gall-bladder neurones that were tested (Fig. 8*A–C*), often resulting in action potential generation (e.g. Fig. 8*A* and *B*). Frequently, discrete synaptic depolarizations were seen up to 20 ms or more after a fibre tract stimulation, indicating that some of these cells receive multiple fast excitatory synaptic input from fibres that have very different conduction times. The fast EPSPs of gall-bladder neurones increased in amplitude when the membrane was hyperpolarized by intrasomal current injection and decreased with direct depolarization. Fast synaptic activity was reversibly abolished in the presence of 100 μM-hexamethonium chloride (Fig. 8*C*), TTX (0·5 μM), or Ca^{2+}-substituted, high-Mg^{2+}

Krebs solution. The amplitude of EPSPs was maintained when fibre tracts were stimulated repeatedly at 3–20 Hz.

Antidromic activation

Occasionally, focal stimulation of fibre tracts with single pulses evoked antidromic spikes (Fig. 8*C*). These responses appeared in an all-or-nothing manner as the

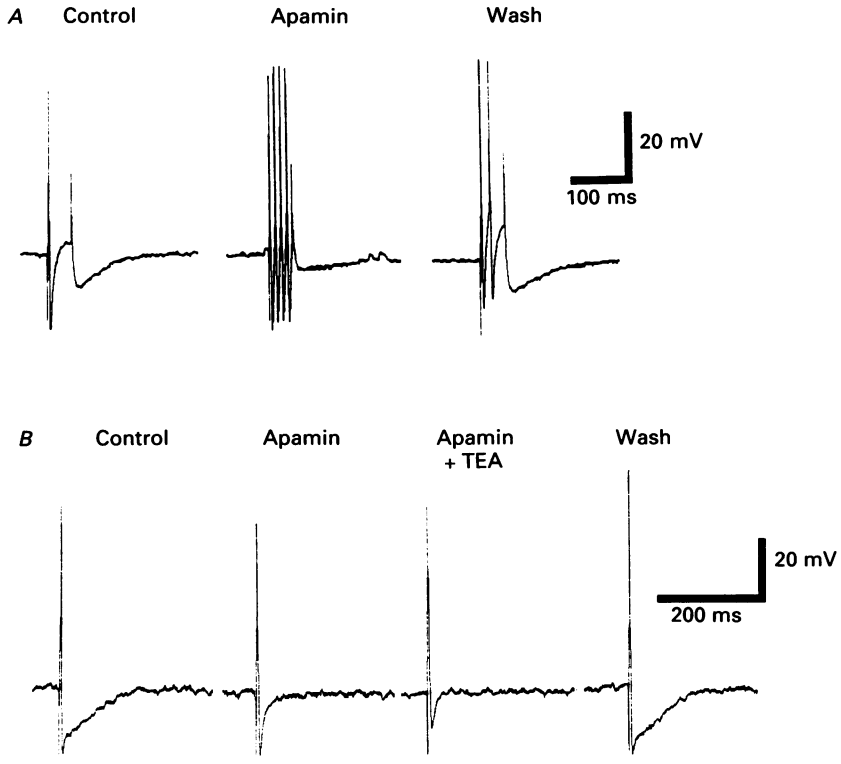

Fig. 7. *A*, in the presence of apamin (10 nM) gall-bladder neurones were capable of firing repetitively throughout a depolarizing current pulse. *B*, effect of TEA when the AHP had been shortened by apamin (10 nM). TEA (1 mM) decreased the amplitude. Resting potentials were: *A*, −52 mV; *B*, −52 mV.

stimulus intensity was increased, and were unaffected by hexamethonium (Fig. 8*C*). The activation of antidromic spikes by stimulation of interganglionic fibre tracts is not surprising since axons of neurones labelled by intracellular injection of HRP were found to pass into fibre tracts.

Slow EPSPs

Prolonged depolarizations and/or prolonged increases in excitability occurred in fifteen of the forty neurones tested following 3–6 s trains of high-frequency (10–20 Hz, 0·5 ms/pulse) fibre tract stimulation. Mean peak amplitude of depolarizations was $5{\cdot}9 \pm 1{\cdot}4$ mV, and the mean duration from the end of the stimulus train to the return of the membrane potential to its resting level was $60{\cdot}6 + 10{\cdot}3$ ms.

These prolonged depolarizations were usually associated with a decrease in input resistance that was reflected by a decrease in the amplitude of electrotonic potentials produced by injection of hyperpolarizing current pulses (Fig. 8*D*). In some cells, following a train of fibre tract stimulation, antidromic responses appeared at the

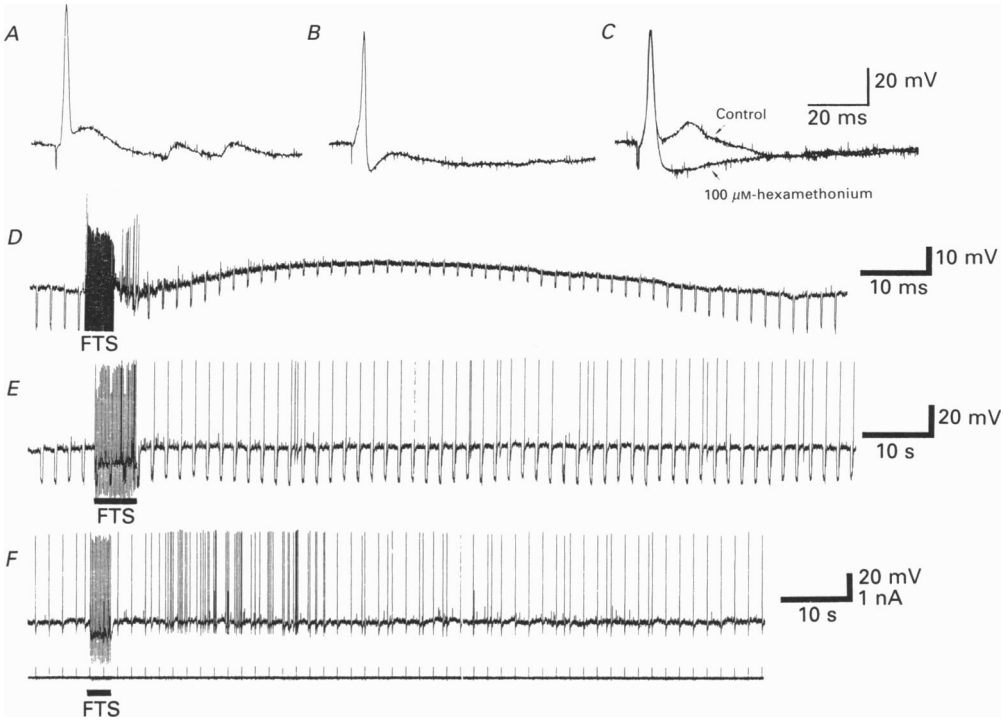

Fig. 8. Fibre tract stimulation (FTS) elicited fast and slow excitatory postsynaptic responses, as well as antidromic responses, in gall-bladder neurones. *A* and *B*, fast synaptic responses to single stimuli of interganglionic fibre tracts. *C*, antidromic response persisted in the presence of hexamethonium, whereas later synaptic responses were blocked by hexamethonium. *D*, a prolonged depolarization with an associated decrease in input resistance was elicited by a train of 5 s at 20 Hz. *E*, increase in excitability of a gall-bladder neurone, represented by the onset of anodal break action potentials, was seen following a repetitive stimulus (6 s at 10 Hz). *F*, an increase in spontaneous activity was seen following a train of stimulus (3 s at 10 Hz). Resting potentials were: *A*, −54 mV; *B*, −50 mV; *C*, −48 mV; *D*, −50 mV; *E*, −51 mV.

offset of hyperpolarizing current pulses (Fig. 8*E*). Increases in spontaneous activity were also observed following trains of high-frequency fibre tract stimulation in some cells (Fig. 8*F*). In all four cells tested, superfusion of the preparation with a calcium-free solution abolished the slow depolarizing response. Atropine (2 μM) did not block the slow depolarization ($n = 3$).

Responses to 5-HT, substance P and bethanechol

Microejection of 5-HT (1 mM) onto gall-bladder neurones resulted in a prolonged depolarization that was associated with a decrease in input resistance (Fig. 9*A*). The responses to 5-HT were dependent on the duration of the microejection of 5-HT.

Depolarizations were observed in four of eight neurones tested and averaged $11{\cdot}4 \pm 1{\cdot}4$ mV in amplitude and $117 \pm 8{\cdot}5$ s in duration (calculated maximum responses for each neurone). These responses to 5-HT had reversal potentials in the range of -5 to 0 mV. Application of 5-HT resulted in a brief hyperpolarization in

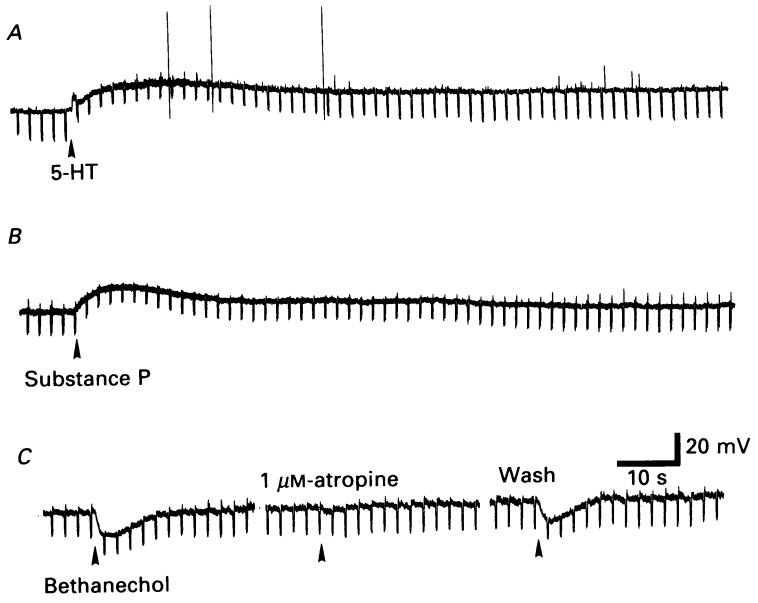

Fig. 9. Effects of 5-HT, substance P and bethanechol on the membrane potential and input resistance of gall-bladder neurones. *A* and *B*, microejection of both 5-HT (*A*; 1·0 mM, 400 ms at 140 N/m^2) and substance P (*B*; 0·1 mM, 800 ms at 140 N/m^2) caused a prolonged depolarization that was associated with decreased input resistance. *C*, microejection of bethanechol (1·5 s at 140 N/m^2) resulted in a hyperpolarization of the membrane that was associated with a decrease in input resistance. The response to bethanechol was blocked by atropine (middle trace), and recovered after a 20 min wash (right trace). Application of the pressure-ejection pulse is indicated by the arrow-head. Resting potentials were: *A*, -50 mV; *B*, -52 mV; *C*, -46 mV.

two of the other neurones that were tested and no response was observed in the other two neurones upon application of 5-HT.

Microejection of substance P onto gall-bladder neurones resulted in duration-dependent responses that were similar to the depolarizing responses seen following application of 5-HT. Application of substance P resulted in a depolarization that was associated with a decrease in input resistance (Fig. 9*B*). Depolarizations were observed in all eight neurones tested and averaged $11{\cdot}7 \pm 1{\cdot}8$ mV in amplitude and had durations of $79 \pm 22{\cdot}3$ s. The depolarization caused by substance P decreased in amplitude when the membrane was depolarized and increased when the membrane was hyperpolarized, in the potential range of -10 to -70 mV.

When bethanechol was microejected onto neurones, a hyperpolarization that was associated with a decrease in input resistance was observed (Fig. 9*C*). Hyperpolarizations, averaging $6{\cdot}3 \pm 1{\cdot}3$ mV in amplitude and $7{\cdot}3 \pm 0{\cdot}8$ s in duration, were observed in six of nine cells that were tested. In two of these cells a biphasic response

was observed; the hyperpolarization was followed by a prolonged depolarization of approximately 2·5 mV in amplitude. Three of the nine cells that were tested did not respond at all. The hyperpolarization caused by bethanecol increased in amplitude when the membrane was depolarized and decreased when the membrane was hyperpolarized, in the potential range of −40 to −60 mV. Responses to bethanechol were reversibly blocked by atropine (1–2 μM) in all four cells that were tested (Fig. 9*C*).

DISCUSSION

The results of this study clearly demonstrate that a single class of neurone is present in the guinea-pig gall-bladder, based on their structure and on the electrophysiological characteristics of their passive properties and evoked responses. Further, the properties of the guinea-pig gall-bladder neurones do not correspond to those described for the classes of neurones in the small intestine of this species (see Wood, 1987). This is a surprising finding since neurones of the gall-bladder are derived from the same set of precursor cells that give rise to the neurones of the small intestine, and since the morphological complexity of the ganglionated plexus of the gall-bladder is reminiscent of the enteric ganglionated plexuses (Mawe & Gershon, 1989).

Structural properties of gall-bladder neurones

Results from intracellular injections of HRP into single gall-bladder ganglion cells indicated that these neurones have a soma and a single long process that is thought to be an axon, but no discernible dendritic arborization. Neurones of the small intestine (see Costa, Furness & Llewellyn-Smith, 1987) and gastric corpus (Schemann & Wood, 1989), on the other hand, are of variable shape and size. Some have numerous long thin processes (Dogiel type II cells) and others have a single long thin process and several short club-like projections from the soma (Dogiel type I cells). The neurones of the gall-bladder appeared more similar to neurones that have been described in the submandibular ganglion of the guinea-pig, which have rudimentary dendritic arbors at most (Snider, 1987).

Conductances that underlie the action potential

The depolarizing phase of the action potential of gall-bladder neurones is primarily the result of inward Na^+ currents, because TTX completely blocked the action potentials. The appearance of a calcium-dependent spike during superfusion with TTX and TEA indicates the presence of a voltage-dependent calcium conductance in the rising phase of the spike as well. A similar phenomenon was described by Schemann & Wood (1989) for gastric corpus neurones of the guinea-pig. It is probable that blockade of a calcium-activated potassium conductance and/or the delayed rectifier by high concentrations of TEA allows for an opening of voltage-sensitive calcium channels. Activation of such channels could be responsible for the appearance of spikes when TEA is added to TTX solution, and for the shoulder observed in the falling phase of broadened spikes in the presence of TEA alone. Neither low concentrations of TEA nor 3,4-DAP, by themselves, significantly altered

the falling phase of the spike, but when they were applied together the spike was broadened. These two currents are commonly associated with the repolarizing phase of neuronal action potentials (see Castle, Haylett & Jenkinson, 1989).

After-hyperpolarizations of gall-bladder neurones are most likely the result of an activation of potassium conductances. This view is supported by the potassium concentration-dependent changes that were observed in the amplitudes and reversal potentials of AHPs in these cells. The AHP of gall-bladder neurones can be divided into an initial phase, of larger amplitude, continuous with the repolarization of an action potential, and a later phase of progressively declining amplitude. The potassium conductances that are involved in both phases of the AHP are probably calcium-dependent since a significant decrease in both amplitude and duration of the AHP was seen in calcium-free, high-magnesium solutions.

It is likely that at least two distinct calcium-dependent potassium conductances are responsible for generating the two phases of the AHP. TEA decreased the initial fast component of the AHP, but did not alter the duration. Apamin, as well as curare, decreased the duration of the AHP, but did not affect the amplitude. Considering the pharmacological distinctions that exist amongst potassium channels, the data from this study indicate that an opening of SK channels may be responsible for the later phase of the AHP. This type of channel is calcium-activated and it is sensitive to apamin and curare (see Castle *et al.* 1989). The initial phase of the AHP may be associated with the opening of the BK potassium channels. The BK potassium channel is calcium-activated, it is sensitive to TEA (see Castle *et al.* 1989) and it is *not* sensitive to 3,4-DAP (Goh, Kelly & Pennefather, 1989). The initial phase of the AHP was not affected by 3,4-DAP alone, but when TEA was added to the bath as well, the amplitude of the initial phase of the AHP was decreased. It is doubtful that the delayed rectifier potassium current is responsible for the initial phase of the AHP since this current is not calcium-dependent, and since 3,4-DAP would have decreased the amplitude of the AHP since it selectively blocks the delayed rectifier current (Goh *et al.* 1989).

The later phase of the AHP appears to be a crucial limiter of the firing properties of these cells. Normally, gall-bladder neurones have phasic firing properties; decreasing the duration of the AHP with apamin, curare or Ca^{2+} substitution converted the cells from a phasic state to a tonic state.

Synaptic activity

This study indicates that fast EPSPs, mediated by nicotinic cholinergic receptors, comprise a major component of the synaptic input that is received by gall-bladder neurones. In addition, many neurones of the gall-bladder receive slow excitatory synaptic input. A recent study of projections to the gall-bladder (Mawe & Gershon, 1989) indicates that there are several possible sources of synaptic input onto ganglion cells of the guinea-pig gall-bladder. They include vagal efferent fibres from the dorsal motor nucleus of the vagus, neurones of the coeliac ganglion and myenteric neurones of the duodenum that project to the gall-bladder. An additional possible source of synaptic input to gall-bladder neurones is projections from other ganglia within the gall-bladder. Intracellular injections of HRP, in the present study, revealed axons from gall-bladder neurones passing through fibre tracts to other ganglia.

Slow depolarizations, that were associated with a decrease in input resistance, were evoked in about one-third of the neurones that were tested with trains of high-frequency stimulation of fibre tracts. These depolarizations appear to be synaptic since they were inhibited in a calcium-free, high-magnesium solution. In the small and large intestines, slow EPSPs are associated with an increase in input resistance that is due to a decrease in a calcium-mediated potassium conductance. Type II/AH cells of the small intestine have an on-going calcium-mediated potassium conductance that contributes to the relatively negative resting membrane potential of these cells (Grafe, Mayer & Wood, 1980). Reduction of this conductance in type II/AH cells results in a depolarization of the membrane with an associated increase in input resistance. In the case of gall-bladder neurones, neither the membrane potential nor the input resistance was altered by TEA, apamin, curare or the removal of calcium from the superfusion solution. This indicates that there is not a significant on-going contribution of calcium-mediated potassium conductances to the resting membrane potential in these cells, and that these cells must be depolarized through some other mechanism. One potential mechanism is an activation of non-specific cation channels.

5-Hydroxytryptamine and substance P are two possible mediators of slow EPSPs in the ganglionated plexus of the gall-bladder. Both 5-HT (Mawe & Gershon, 1989) and substance P (Goehler, Sternini & Brecha, 1988; Mawe & Gershon, 1989) have been shown to be present in this system. When 5-HT or substance P was applied to neurones of the gall-bladder in the present study, responses similar to the slow EPSPs were observed. Prolonged depolarizations associated with a decrease in input resistance occurred. It is doubtful that slow EPSPs in the ganglionated plexus of the guinea-pig gall-bladder are cholinergic. The most prominent and consistent response to the muscarinic agonist, bethanechol, was a hyperpolarization rather than a depolarization. In addition, slow EPSPs were not blocked by the muscarinic antagonist atropine.

Concluding remarks

These experiments have provided a preliminary characterization of neurones in the wall of the guinea-pig gall-bladder. These are rather homogenous in their properties, which differ in certain respects from the properties of intramural neurones of the stomach or intestine. All neurones receive nicotinic synaptic input; neurones with the properties of type I/S cells or type II/AH cells were not seen. Responses to 5-HT and substance P were depolarizations associated with a conductance increase, rather than the conductance decrease often seen in neurones of the small bowel, and a muscarinic agonist, which depolarizes intestinal neurones, caused a hyperpolarization of gall-bladder neurones.

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