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EXTRAGLANDULAR INNERVATION OF THE SALIVARY CELLS OF THE LEECH *HAEMENTERIA GHILIANII*: NEURONAL STIMULATION ELICITS GLAND-CELL ACTION POTENTIALS AND SECRETION

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Summary

1. Each salivary gland cell of *Haementeria* extends a single process, or ductule, anteriorly into the proboscis; secretory products are released at the ductule ending. Some ductules secrete into the lumen of the proboscis and others at the outer surface of its tip, more than 5 cm from the gland in large leeches.

2. Depolarization of a gland-cell body elicits action potentials which appear to be conducted along the ductule to its ending. Electrical stimulation of the proboscis tip elicits action potentials in those ductules which end there, and the impulses are propagated to the cell body (approx. 5 cm s^{-1}).

3. Bathing the salivary glands in calcium-free saline causes spontaneous repetitive firing in the cell bodies and also elicits secretion at the proboscis tip (bathed in normal saline); the action potential thus appears to be a stimulus for secretion.

4. A paired stomatogastric nerve, from the brain, enters the proboscis near its base. Cobalt-filling of the nerve shows numerous cell bodies in the brain and first body ganglion, and an intricate network of fibres and a cluster of stained cell bodies near its entry point in the proboscis.

5. Repetitive stimulation of the stomatogastric nerve produces action potentials in certain gland cells, after a delay of at least 15 s, and also elicits secretion. The action potentials are initiated near the ductule tip, and are conducted to the cell body. The salivary glands themselves do not appear to be innervated.

6. Application of acetylcholine (ACh), dopamine or octopamine (10^{-4} mol l^{-1}) does not initiate secretion. Neither dopamine nor octopamine excites the gland cells but ACh produces a transient suprathreshold depolarization of the cell body and occasionally elicits 1–3 ductule spikes when applied to the proboscis tip. 5-Hydroxytryptamine (5-HT) produces secretion when applied to the proboscis but not when applied to the glands alone; it does not excite the cells, indicating that the action potential is not the only stimulus for secretion. 5-HT produces a

Key words: action potential, leech, salivary gland, secretion, serotonin.

depolarization, and increase in membrane resistance, in the cell body, and prevents the rapid adaptation of action potentials which occurs during maintained depolarization.

7. Electrophoretic analysis shows that the protein compositions of secretions at the proboscis tip and in the lumen are completely different, with the tip apparently secreting only two major proteins. These same two protein bands occur in the cytoplasm of certain gland-cell bodies which can be distinguished in living glands on the basis of size and degree of staining with Methylene Blue.

8. Following stimulation of the stomatogastric nerve, secretory products at the proboscis tip can be seen to emerge from discrete points which appear to be single ductule endings. This presents the possibility of studying excitation–secretion coupling in single cells.

Introduction

The salivary cells of the giant leech *Haementeria* are grouped into two pairs of discrete glands which lie at the base of a long, muscular proboscis (Sawyer *et al.* 1982). The two large anterior glands each contain about 240 cells ranging in diameter from 400 to 1200 μm; the smaller posterior glands contain cells of about 100 μm in diameter. Each cell has a single process which leaves the gland and enters the proboscis, and release of secretory products appears to occur at the end of each process or 'ductule'. Some ductules do not travel far from the gland whereas others reach the tip of the proboscis, a distance of more than 5 cm in large leeches.

The proboscis–salivary gland complex is supplied by a paired stomatogastric nerve from the brain, which enters near the proboscis base close to the attachment of the salivary glands. The gland-cell bodies produce overshooting, calcium-dependent action potentials in response to depolarizing current applied through an intracellular microelectrode (Marshall & Lent, 1984; Jones *et al.* 1985; Wuttke & Berry, 1988). We report here that the gland cells are also activated by stimulation of the stomatogastric nerve but that the innervation of each cell is outside the gland, close to the ductule tip; impulses travel along the ductule from the tip into the cell body in the gland.

Materials and methods

Specimens of *Haementeria ghilianii* were obtained from a breeding colony held at Biopharm (UK) Ltd, Swansea, where they were maintained in aquaria at 26°C. The aquarium water contained (mmol l^{-1}): NaCl, 0·63; $CaCl_2$, 0·07; $MgCl_2$ 0·07; K_2SO_4, 0·01; Tris maleate, 1 (pH 6·0) (Sawyer *et al.* 1981).

Preparation

The proboscis and attached salivary glands, together with the brain and first few ganglia of the ventral nerve cord, were removed from the animal after cutting through the oesophagus. The preparation (see Fig. 1) was pinned to stretch th

two sheaths which surround the proboscis (the sheaths are attached to the proboscis only at its base). The stomatogastric nerve, which runs between the sheaths from the brain to the base of the proboscis, was freed by cutting the outer sheath. The preparation was then transferred to a Perspex experimental bath (volume 1 ml) where it was pinned to a layer of Sylgard. Careful pinning was essential to avoid the dislodging of microelectrodes in gland cells by movements of the proboscis; pins were placed through the inner sheath and also through the endothelium which invests the salivary glands. Physiological saline flowed through the bath at about 3 ml min^{-1}; its composition (mmol l^{-1}) was: NaCl, 125; KCl, 4; $CaCl_2$, 1·8; glucose, 11; Tris maleate, 10 (pH 7·4) at room temperature (18–22 °C).

Electrophysiology

The techniques were similar to those described by Wuttke & Berry (1988). Briefly, individual gland cells were impaled with a bevelled, KCl^-filled microelectrode (10–20 MΩ) for recording membrane potential. A second electrode was sometimes inserted for passing current. Each electrode was connected to a Digitimer NL 102 amplifier, and signals were monitored on a pen recorder (Brush 2200S) and stored on tape (Thorn EMI 3000 FM tape recorder). Glass-tipped suction electrodes were used for stimulating the stomatogastric nerve or the tip of the proboscis.

Effects of some putative transmitters were examined by perfusion at known concentration, using a multiway valve (Holder & Sattelle, 1972). The following substances were applied: acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, dopamine hydrochloride and octopamine hydrochloride (Sigma).

Cobalt filling of the stomatogastric nerve

The stomatogastric nerve was filled with cobalt to trace its pathways in the proboscis and in the brain. In isolated preparations of (a) supra- and sub-oesophageal ganglia plus the first few ganglia of the nerve cord, and (b) the proboscis and attached salivary glands, the cut end of the stomatogastric nerve was placed into a Vaseline trough filled with distilled water. The rest of the preparation was covered with saline containing 18 mmol l^{-1} $MgCl_2$ to relax the nerve, which otherwise tended to contract and pull out of the trough (there are muscle fibres in the sheaths of nerve trunks and of ganglia). The length of nerve was kept as short as possible to reduce diffusion distance. After 5 min the distilled water was replaced by aqueous 5 % cobalt chloride containing bovine serum albumin (0·013 g 100 ml^{-1} solution; Strausfeld & Obermayer, 1976) or aqueous 6 % hexammine cobalt (III) chloride (Sigma). The trough was closed with Vaseline and more saline added to the rest of the preparation. Preparations were kept at 8 °C for 12–24 h (brain) or up to 44 h (proboscis).

The cobalt was subsequently precipitated for 15–30 min in 1 % sodium sulphide (w/v) in physiological saline (pH 7·4) and the preparation then washed in distilled water and fixed in Carnoy's fixative. After silver intensification (Bacon & Altman,

1977) tissues were dehydrated in ethanol, cleared in methyl salicylate and embedded in Ralmount.

Analysis of secretions

Secretory products are released at the outer surface of the proboscis tip and in the proboscis lumen. These secretions were separately sucked into a fine syringe needle and their proteins were resolved by SDS polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970), using a Pharmacia Phast System and gradient gels (10–15 %). The following relative molecular weight markers (Pharmacia) were co-electrophoresed: phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; α-lactalbumin, 14 400. Gels were subsequently stained with silver (Heukeshoven & Dernick, 1985).

In addition, individual gland-cell bodies were ruptured to extrude their cytoplasm which was collected in a syringe needle and subsequently analysed as above.

Results

General features of the preparation

The preparation consisted of the proboscis and attached salivary glands, the brain and first few body ganglia, and the paired stomatogastric nerve which runs between two sheaths and enters the proboscis near its base (Fig. 1). The inner sheath encloses the proboscis and is attached at one end to its base and at the other (in the intact animal) to the mouth. The proboscis lies freely within this sheath and is able to extend several centimetres beyond its opening.

For further details of the anatomy see Sawyer *et al.* (1982) and Sawyer (1986).

Cobalt-filled stomatogastric nerve

Central projections

When the stomatogastric nerve was back-filled with cobalt chloride its two main trunks were found to enter the brain and branch into the nerves to the mouth; processes also ran anteriorly into the sheath but there were no stained cell bodies within the brain. With hexammine cobalt chloride, however, extremely fine processes could be seen coming from the main trunks, and cell bodies in the brain and first segmental ganglion were stained. Some of the cells, which apparently form bilaterally symmetrical pairs, seem large enough to be identifiable and recorded with microelectrodes (Fig. 2). No processes were seen to pass back to other ganglia of the ventral nerve cord.

Peripheral projections

The two stomatogastric nerve trunks enter the proboscis near its base and each runs forward towards the tip. They could be traced for several millimetres bu

disappeared well before reaching the end of the proboscis. Processes could also be traced posteriorly to the level of the salivary glands but it was not possible to determine whether they entered the glands. Silver intensification was necessary to follow them this far and the procedure disrupted the glands. At the entry point of the stomatogastric nerve into the proboscis there is an intricate network of stained fibres and a cluster of stained cell bodies which lie in the inner sheath (Fig. 3). These cell bodies are not visible in living, unstained preparations but cells with the same characteristic pattern of distribution are clearly revealed by Methylene Blue. They do not take up Neutral Red, which tends to stain monoaminergic neurones (Lent, 1981).

The stomatogastric nerve thus contains axons of both central and peripheral neurones.

Propagated action potentials in the ductules

Various attempts were made to determine whether the action potentials which are produced in gland-cell bodies by applied depolarizing current (see Fig. 11) are conducted along the ductules. It would be extremely difficult to penetrate ductules with a microelectrode once they have entered the proboscis because they disappear amongst the musculature. Also, the proboscis produces continuous movements which may dislodge an electrode even from a cell body in a well-secured gland. Ductules are also difficult to trace within the gland itself because they form its core, surrounded by the cell bodies. Some ductules come close to the surface at various points, and these could be impaled and shown to produce action potentials. In no case, however, was it possible to trace a ductule for more than 3 mm from its cell body.

Extracellular recordings, using a suction electrode attached to the tip of the proboscis, failed to reveal any potential changes associated with spikes elicited in a cell body. If the tip of the proboscis was stimulated by current pulses, however, a subthreshold depolarizing response occurred in certain cells after each stimulus (Fig. 4A). These responses followed stimuli on a one-to-one basis up to frequencies of $15\,min^{-1}$ (Fig. 4B) although in some cells there were intermittent failures. Occasionally they disappeared altogether but could be reinstated by a brief period of high-frequency stimulation (Fig. 4B). The responses appear to represent invasion of the cell body by action potentials in the ductule, propagated from the tip. Evidence is presented below that the cell bodies are not innervated, and that these brief depolarizing responses are not synaptic potentials. In the following account they will be referred to as 'ductule spikes', i.e. intrasomatically recorded potentials which are produced by electrotonic spread of current associated with an action potential in the ductule. Their relatively small size presumably results from the large difference between membrane area of the ductule and of its cell body (ductules have a diameter of about 30 μm in the gland whereas cell bodies range up to 1200 μm and their membranes are greatly invaginated; Walz *et al.* 1988).

The typical configuration of a ductule spike recorded in the cell body is shown in

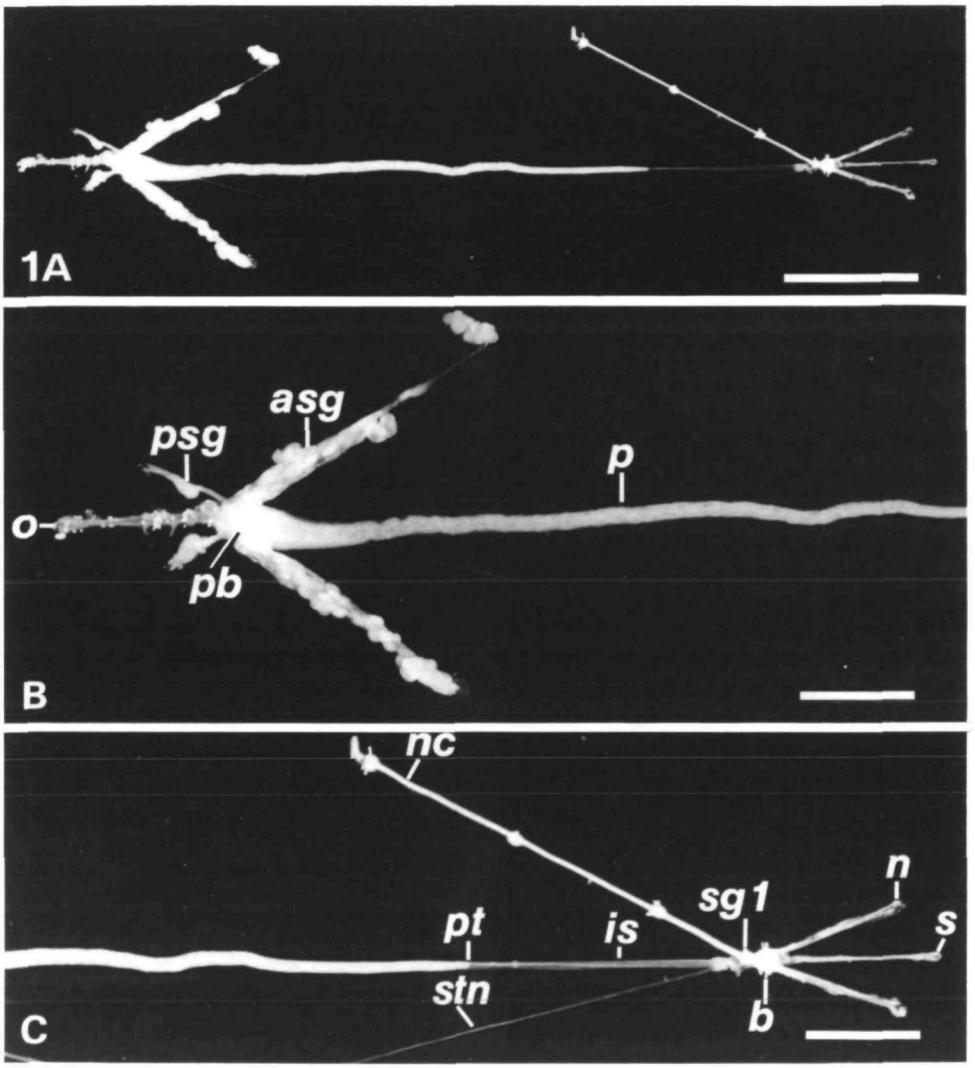

Fig. 4A. Amplitudes ranged from 2 to 30 mV, occasionally reaching spike threshold for the soma. In some cells the amplitude remained constant during repetitive activation whereas in others there was wide variation. There was usually a small undershoot (0·5–2 mV). The latency of the response was 1–2 s. It was difficult to estimate conduction velocity because ductules are coiled in the proboscis, but values of 5 cm s^{-1} were obtained using the length of a fully stretched proboscis. Similar responses, with shorter latency, could be obtained after removing much of the proboscis and stimulating the remaining cut end. It was not possible to elicit responses to tip stimulation in all cells investigated, however, presumably because their ductule terminated nearer the base of the proboscis. All stimuli produced contractions of the proboscis.

Three lines of evidence indicate that the responses to stimulation of th

Fig. 1. Photographs of the isolated preparation. A shows the entire preparation, B and C show higher magnifications of the anterior and posterior ends (ventral surface uppermost). The proboscis lies freely within a double sheath. The outer sheath has been removed to display the stomatogastric nerve which is displaced downwards for clarity; the nerve runs from the brain between the two sheaths and enters the proboscis at its base (there are two separate trunks within the nerve). Three pairs of brain nerves have been cut so that only the stumps remain. In this preparation the proboscis is partially contracted but may elongate and extend several centimetres beyond the opening of the inner sheath. This sheath passes through the brain and normally encompasses the buccal cavity which opens to the outside at the mouth. *asg*, anterior salivary gland; *b*, brain; *is*, inner sheath; *n*, nerves to the mouth; *nc*, nerve cord; *o*, oesophagus; *p*, proboscis; *pb*, proboscis base; *psg*, posterior salivary gland; *pt*, proboscis tip; *s*, sheath; *stn*, stomatogastric nerve; *sg 1*, first segmental ganglion. Scale bars, A 1 cm, B,C 5 mm.

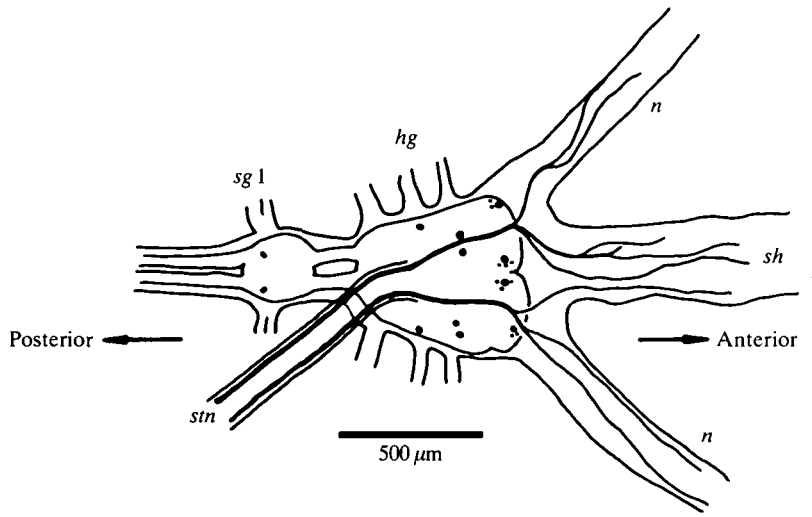

Fig. 2. *Camera-lucida* drawing of the brain and first segmental ganglion to illustrate the axons and nerve cell bodies revealed by back-filling the stomatogastric nerve with cobalt. The nerve has two main trunks, separated by connective tissue. *hg*, head ganglion (brain); *n*, nerve to the mouth; *sh*, sheath; *stn*, stomatogastric nerve; *sg 1*, first segmental ganglion.

proboscis tip are not synaptic potentials. (i) They persisted in saline containing 18 mmol l^{-1} Mg^{2+} to block synaptic transmission (Fig. 5). This high Mg^{2+} concentration prevented contraction of the proboscis produced by stimulation of the stomatogastric nerve. (ii) The responses were not potentiated by hyperpolarizing the gland cell with applied current; in some cells hyperpolarization to −110 mV produced little or no change in amplitude, and in others the response became much reduced (Fig. 6). The possibility was considered that they were synaptic potentials produced by a decrease in conductance, but there was no inversion even at −120 mV. The decrease in amplitude with hyperpolarization presumably

resulted from a block of the incoming ductule spike further from the cell body. (iii) The responses could be blocked by collision with intrasomatically evoked impulses travelling in the opposite direction (Fig. 7). This indicates that they were produced by ductule spikes conducted from the tip (Parnas *et al.* 1969). An alternative explanation for these results is that the responses are EPSPs produced by a nonrectifying electrotonic synapse, but this is extremely unlikely: no gland cells are known to receive such input, and it would probably be ineffective in such large cells.

The ionic mechanism of ductule spikes was determined by substitution of extracellular sodium and by use of cobalt to block calcium channels. Action potentials in the cell body are calcium-dependent: they are unaffected by removal of external sodium and are reversibly blocked by 5 mmol l^{-1} cobalt (Marshall & Lent, 1984; Jones *et al.* 1985; Wuttke & Berry, 1988). Ductule spikes recorded in the cell body were not blocked by sodium removal (Fig. 8; sodium was replaced by *N*-methyl-D-glucamine). There was, however, an increase in the number of failures, whose cause is unknown; it did not appear to be related to the transient hyperpolarization produced by Na^+ removal because failures continued to occur during the return to the original membrane potential, though the time course of the change in membrane potential could be different in the ductule. Addition of

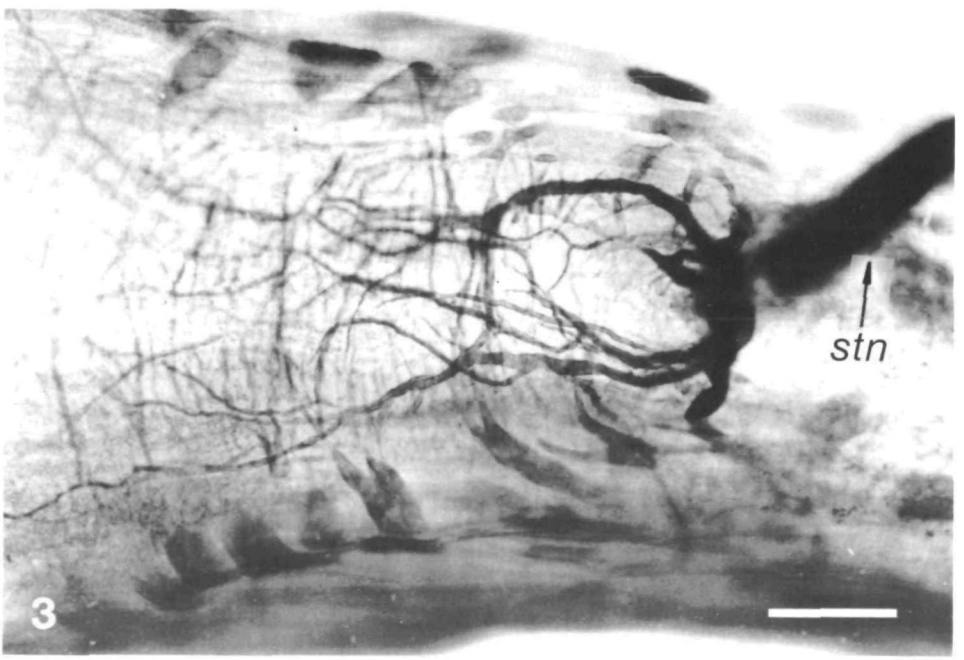

Fig. 3. Cobalt-filled stomatogastric nerve at its entry into the proboscis. The stained nerve cell bodies lie in the inner sheath, and form a characteristic pattern encircling the proboscis. They can be seen in living preparations by using Methylene Blue but they do not take up Neutral Red. Anterior is to the left. *stn*, stomatogastric nerve. Scale bar, 100 μm.

5 mmol l^{-1} cobalt usually produced intermittent failures of response rather than a complete block. Responses were reversibly abolished by 10 mmol l^{-1} cobalt but showed a slow recovery when cobalt was washed out (approx. 20 min) whereas recovery of somatic action potentials from 5 mmol l^{-1} cobalt takes only a few minutes (Wuttke & Berry, 1988). These differences in cobalt concentration and

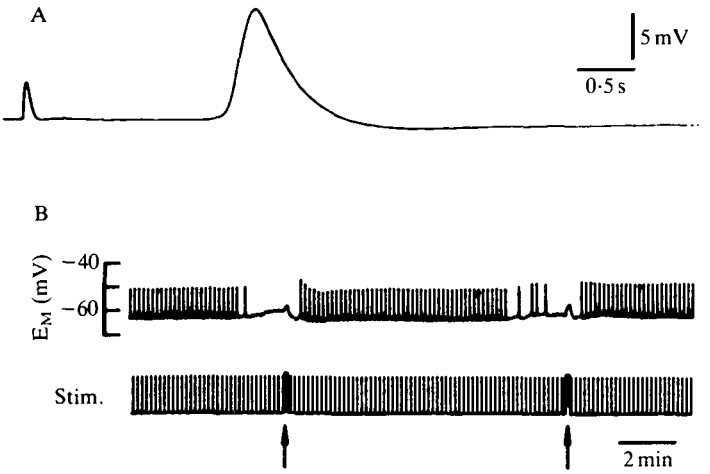

Fig. 4. (A) Response of a gland-cell body to a single electrical stimulus applied to the proboscis tip (2 ms, 5 V square pulse; the small deflection is the stimulus artefact). (B) Responses (upper trace) follow stimuli to the proboscis tip (bottom trace) one-for-one at frequencies of 6 min^{-1}. On two occasions the responses disappeared but were reinstated by a brief period of high-frequency stimulation (10 s^{-1}, arrows; the apparent depolarizations are stimulus artefacts). Responses appeared at a certain stimulus intensity and were not altered by increasing the intensity. These subthreshold depolarizing potentials appear to represent invasion of the cell body by action potentials in the ductules (propagated from the tip) and are hereafter referred to as ductule spikes.

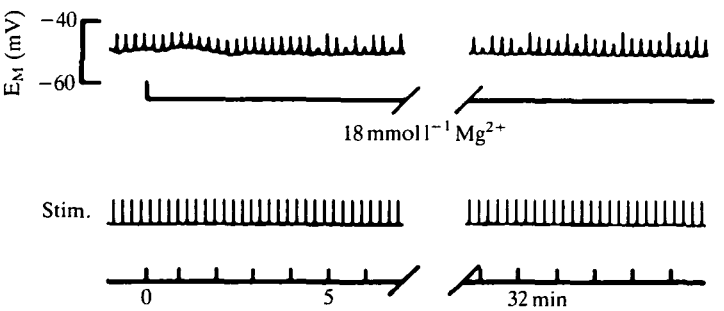

Fig. 5. Ductule spikes (top trace) elicited by electrical stimulation of the proboscis tip are not blocked by 18 mmol l^{-1} Mg^{2+} which blocks synaptic transmission (e.g. Mg^{2+} eliminated contractions of the proboscis produced by stimulating the stomatogastric nerve). In this cell there were occasional failures of response in high-Mg^{2+} saline which may result from the effect of this ion on spike threshold. Intermittent failures occurred in certain other cells, however, bathed in normal saline.

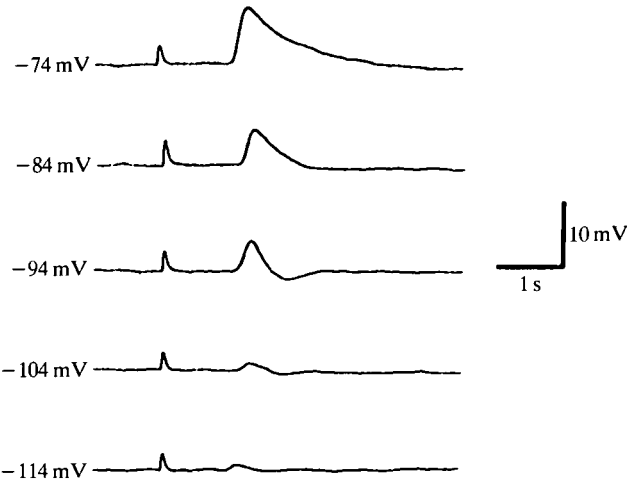

Fig. 6. The depolarizing response in a gland-cell body to stimulation of the proboscis tip is reduced by hyperpolarization. Each trace shows a response (preceded by the stimulus artefact) recorded at different levels of membrane potential (resting membrane potential, −74 mV; hyperpolarizing current was passed through a separate electrode). Some cells showed little change in response with hyperpolarization, and no cell showed a potentiation. This indicates that the responses are not synaptic potentials but represent invasion of the soma by action potentials conducted along the ductule.

recovery time may be due to more restricted access of solutions to the ductules which are surrounded by muscle fibres in the proboscis. The results indicate that the ductule spike is Ca^{2+}-dependent but they are not as clear-cut as those for the soma spike.

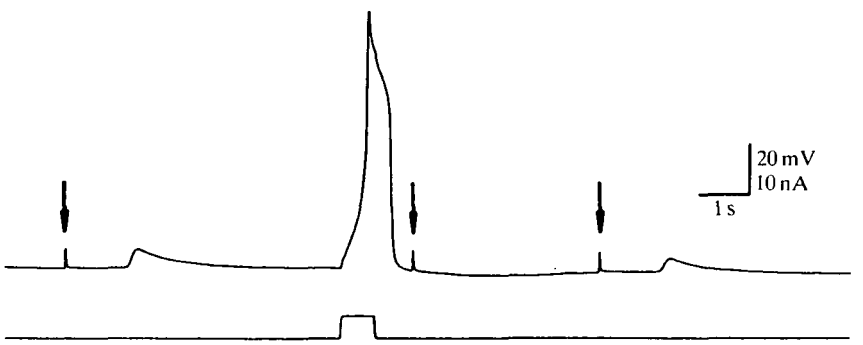

Fig. 7. Collision experiment to determine whether the depolarizing response in a gland-cell body to stimulation of the proboscis tip is produced by synaptic input or by an action potential conducted along the ductule. Three stimuli were applied to the proboscis (arrows indicate stimulus artefacts). The first and third stimuli were followed by depolarizing responses whereas the second, which was preceded by a somatic spike (evoked by depolarizing current, lower trace) produced no response. This result is expected if the response to tip stimulation is produced by a ductule spike which collides with the soma spike travelling in the opposite direction.

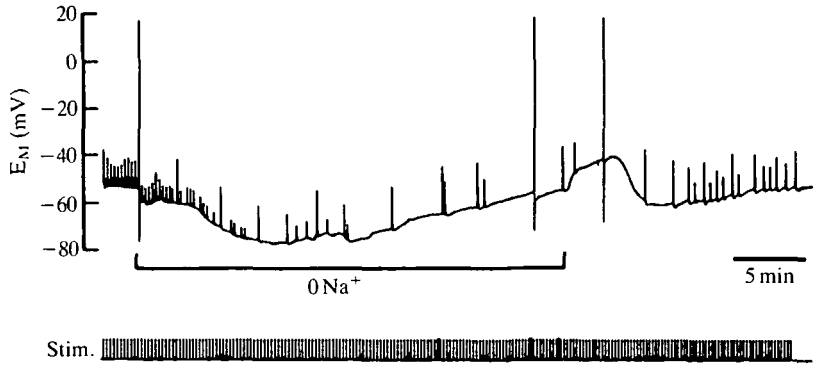

Fig. 8. Ductule spikes (upper trace) elicited by stimulation of the proboscis tip are not blocked by removal of sodium. Substitution of sodium by *N*-methyl-D-glucamine produced the usual transient hyperpolarization (Wuttke & Berry, 1988) during which the ductule spikes became less frequent but did not disappear. Four periods of high-frequency stimulation failed to reinstate the one-for-one following (cf. Fig. 4). Sodium removal rapidly abolished the proboscis contractions produced by stimulating the stomatogastric nerve. Note that on three occasions the ductule spikes reached threshold for the soma.

Responses of gland-cell somata to nerve stimulation

Gland cells were never observed to produce spontaneous action potentials or show signs of spontaneous synaptic input, even in preparations where the brain and first few body ganglia were left attached. If the stomatogastric nerve was stimulated electrically, single stimuli produced a contraction of the proboscis (and of the stomatogastric nerve itself) but no effect on salivary cell bodies. With repetitive stimulation of less than about $10\,s^{-1}$, the gland cells remained inactive while the proboscis became strongly contracted and subsequently produced vigorous peristaltic waves which started near the tip (the orifice at the tip usually remained open). Even without stimulation the proboscis was active; if unrestricted it waved around, became coiled and then uncoiled, and periodically contracted very strongly, but it rarely showed peristalsis. Above about $10\,s^{-1}$, repetitive stimulation of the stomatogastric nerve for 10–20 s produced in some gland cells responses which resembled ductule spikes elicited by stimulation of the proboscis tip (Fig. 9). Evidence is presented below that they are indeed ductule spikes and not synaptic potentials. The responses sometimes stopped after a few seconds until the nerve was stimulated again, and sometimes they continued for several minutes, occurring at frequencies up to $45\,min^{-1}$ (Fig. 10). There was a delay of at least 15 s between the beginning of stimulation and the first response, and delays of 1 min or more were recorded. Similar responses occurred if the nerve was cut and the distal end stimulated. Ductule spikes sometimes reached spike threshold for the cell body (especially the first in a series) but the responses were generally subthreshold. Considerable variation was found between responses to the same stimulation, even in a single cell; this was not correlated with any obvious

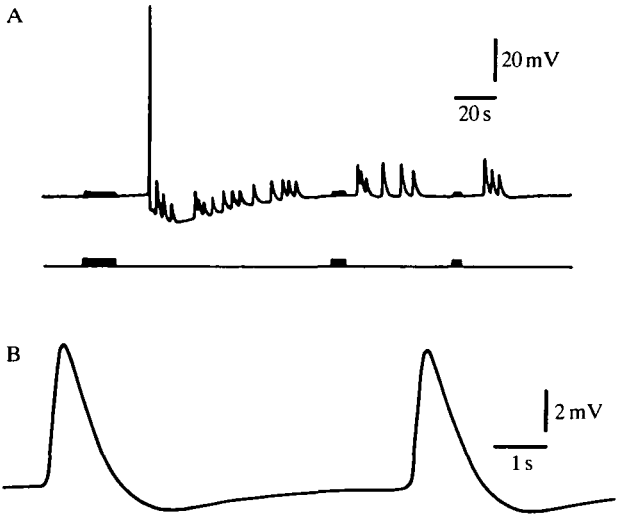

Fig. 9. (A) Repetitive stimulation of the stomatogastric nerve ($12\,s^{-1}$, indicated on the lower trace) produced bursts of responses resembling ductule spikes, recorded in a gland-cell body (upper trace). Evidence is presented that these are indeed ductule spikes, initiated near the ductule tip and conducted to the cell body. The first response triggered a soma spike. (B) Higher speed recording to illustrate the shape of the neuronally evoked responses in the gland-cell body.

movements of the proboscis. Ductule spikes produced by nerve stimulation were abolished by 18 mmol l^{-1} Mg^{2+} after about 20 min but they could still be elicited by stimulation of the proboscis tip after 1 h.

Site of initiation of neurally evoked responses

For a number of reasons, which are explained in the Discussion, it seemed likely that the salivary cells were innervated near the ductule tip rather than in the gland itself. In a cell whose ductule terminated at the end of the proboscis, therefore, the response to nerve stimulation should have been abolished by cutting the proboscis tip. It was indeed found that ductule spikes produced by nerve stimulation disappeared after removing about a 1 mm length from the proboscis tip, whereas stimulation of the cut end of the proboscis continued to produce ductule spikes on a one-for-one basis.

Confirmation that the responses to nerve stimulation were ductule spikes rather than synaptic potentials was more difficult than for the responses to stimulation of the proboscis tip. For example, the absence of a fixed relationship between stimulus and response meant that collision experiments could not be reliably performed. Hyperpolarization of the soma to −100 mV, however, produced little change, or a reduction in amplitude of responses to stimulation of the nerve, providing further evidence that they are not synaptic potentials.

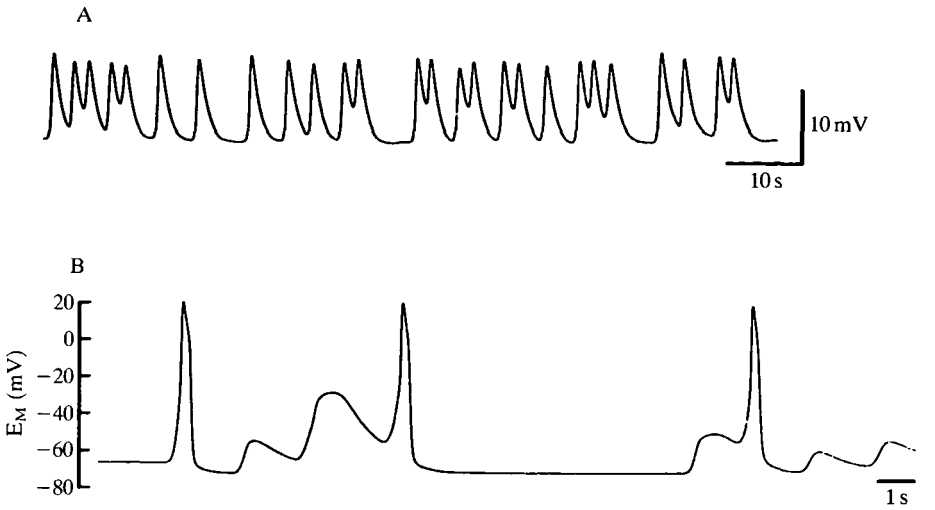

Fig. 10. (A) Recording of ductule spikes in a gland-cell body, following stimulation of the stomatogastric nerve ($10\,s^{-1}$ for 10 s; not shown). Note the relatively high frequency of firing (higher than can be obtained by repetitive stimulation of the proboscis tip or by depolarization of the cell body). The production of spike doublets or triplets is also seen in salivary cells of *Hirudo* which have been stimulated by serotonin (Marshall & Lent, 1988). (B) This shows a rare example of ductule spikes whose effects are additive and reach threshold for the soma. The first ductule spike is sufficiently large to reach threshold alone, and the shape of the third indicates that it produced a local response in the soma.

Responses of gland-cell somata to putative transmitters

The effects of acetylcholine (ACh), 5-hydroxytryptamine (5-HT), dopamine and octopamine were investigated by perfusion ($3\,ml\,min^{-1}$). It was necessary to distinguish between effects on the ductule tip in the proboscis and effects on the cell body in the gland. Recordings from salivary-cell bodies were therefore made in a bath where the proboscis tip alone could be exposed to transmitter by means of a Vaseline partition which separated it from the glands (volume of the compartment containing the tip was 0·5 ml; the presence of a ductule at the tip was confirmed by the presence of a response to tip stimulation).

Application to isolated glands

Of the four transmitter candidates, only ACh elicited spikes. At $10^{-6}\,mol\,l^{-1}$ there was a small subthreshold depolarization but at $10^{-4}\,mol\,l^{-1}$ a large, rapid depolarization resulted in 1–3 action potentials and a large drop in membrane resistance. The effect was transient, however, and membrane potential and resistance returned to normal after about 5 min exposure (Fig. 11).

Depolarizations were also produced by 5-HT, dopamine and octopamine, but these did not reach threshold even at concentrations of $10^{-4}\,mol\,l^{-1}$. A marked effect on excitability was produced by 5-HT (and in some cells by dopamine).

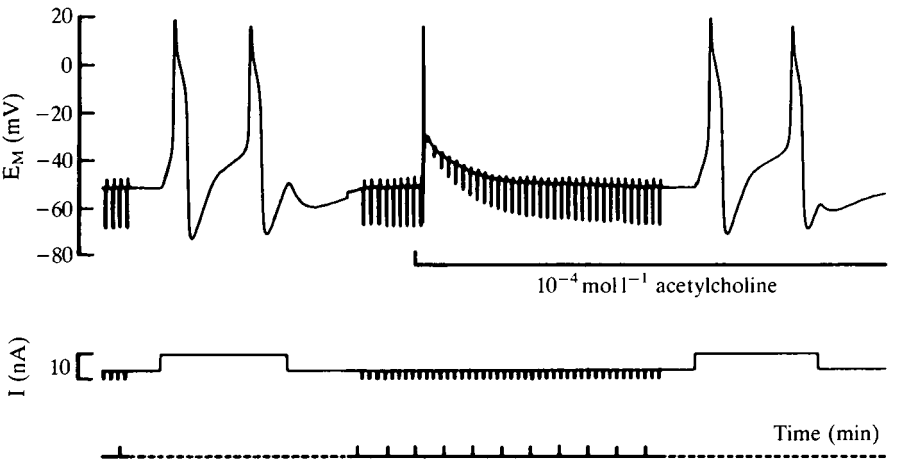

Fig. 11. Application of 10^{-4} mol l^{-1} acetylcholine to the salivary glands produces a transient depolarization and two action potentials in a gland-cell body. Constant-current hyperpolarizing pulses demonstrate an accompanying transient fall in membrane resistance. During the periods indicated by the dashes on the bottom trace, the recording speed was increased 100 times to illustrate the action potentials produced by a depolarizing current pulse. Action potential amplitude and configuration were not affected by acetylcholine.

Fig. 12 shows the typical depolarization and *increase* in membrane resistance produced by 10^{-4} mol l^{-1} 5-HT. Suprathreshold depolarizing current now evoked prolonged spiking whereas before and after 5-HT application the response to the

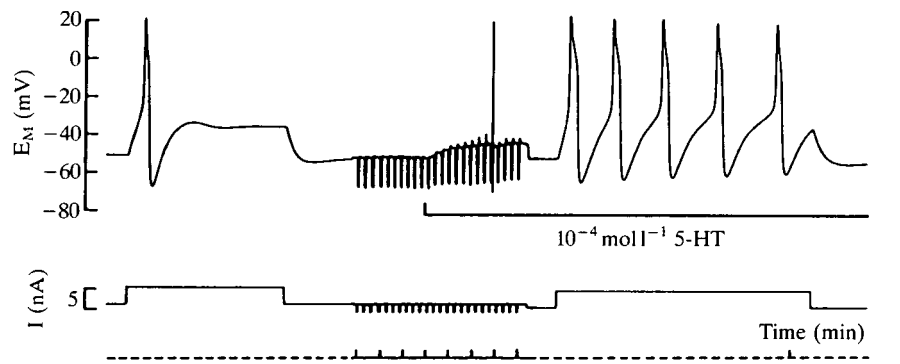

Fig. 12. Application of 10^{-4} mol l^{-1} 5-hydroxytryptamine (5-HT) to the salivary glands produces a subthreshold depolarization and increase in membrane resistance in a gland-cell body. There is now a small rebound depolarization following each hyperpolarizing pulse, and one of these reaches threshold. During the periods indicated by the dashes on the bottom trace, the recording speed was increased 100 times to illustrate action potentials produced by a depolarizing current pulse. The initial response to the depolarizing pulse is a single action potential. In the presence of 5-HT the same current pulse now produces five action potentials (the depolarization produced by 5-HT was first offset by applied current).

same current was a single spike (gland cells rarely produce more than three or four spikes when depolarized by applied current). The influence of 5-HT on excitability is not simply to depolarize the cell towards threshold and thereby increase the effect of the current; in the absence of 5-HT it is impossible to elicit prolonged firing by increasing the current or by applying prior hyperpolarization to remove any inactivation of voltage-dependent channels.

5-HT and dopamine affected the shape of the action potential, tending to produce an increased overshoot, reduced undershoot and increase in duration.

Application to the proboscis tip

5-HT, dopamine and octopamine at concentrations of 10^{-6}–10^{-4} mol l^{-1} produced no effect on the cell body when applied to the proboscis tip. ACh at similar concentrations produced one or two ductule spikes, resembling the effect of application to the cell body but without the transient depolarization (Fig. 13); this effect was not, however, observed in all gland cells tested.

Gland-cell activity and secretion

Some salivary cells secrete into the lumen of the proboscis and others on to its outer surface (Sawyer *et al.* 1982). Little or no secretory product was seen at the surface in unstimulated preparations bathed in physiological saline, but secretion in response to stimulation could be clearly observed. Secretions stuck for a while to the proboscis; they were not readily soluble and dispersed slowly. At high magnification they could be seen to emerge from discrete points which appeared to be single ductule endings. The ductules secrete from their tip directly on to the proboscis surface; they do not discharge first into a common duct (Walz *et al.* 1988; this contrasts with jawed leeches such as *Hirudo*; Marshall & Lent, 1988).

Secretion could be elicited by repetitive stimulation of the stomatogastric nerve, and by exposure of the preparation to 10^{-4} mol l^{-1} 5-HT, but no effect of dopamine, octopamine or ACh was observed. The most powerful stimulant of secretion was calcium-free saline which was applied to the glands alone, with the proboscis in a separate compartment containing the normal concentration of calcium. Removal of calcium elicits spontaneous action potentials in gland-cell bodies, produced by sodium flowing through calcium channels (Wuttke & Berry, 1988). It seems likely that these action potentials are conducted along the ductules and initiate the secretion observed at the proboscis tip.

Composition of secretions

The protein composition of salivary gland extracts has been documented (Budzynski *et al.* 1981*a*,*b*) but little is known of the composition of secreted material. Our interest focused on the secretions at the proboscis tip because they can clearly be seen forming from individual ductules, and thus offer the possibility of studying secretory mechanisms using single cells. Secretion was initiated by repetitive stimulation of the stomatogastric nerve, and material from the proboscis tip and lumen was sucked separately into a fine syringe needle for subsequent

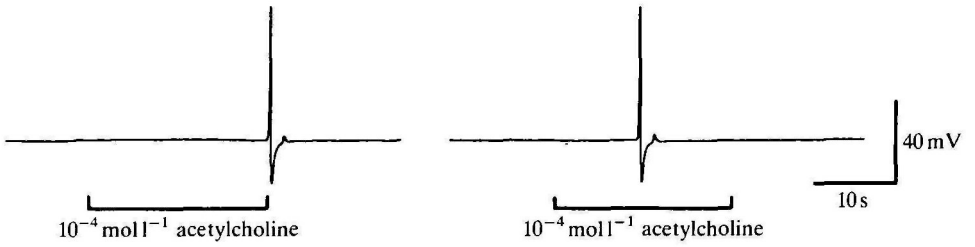

Fig. 13. Application of 10^{-4} mol l^{-1} acetylcholine to the proboscis tip elicits an action potential followed by a small depolarization (ductule spike) in a gland-cell body. The two recordings are from the same cell to illustrate the consistent nature of the response (the different latencies probably result from different positions of the proboscis tip in the bath, caused by proboscis movements).

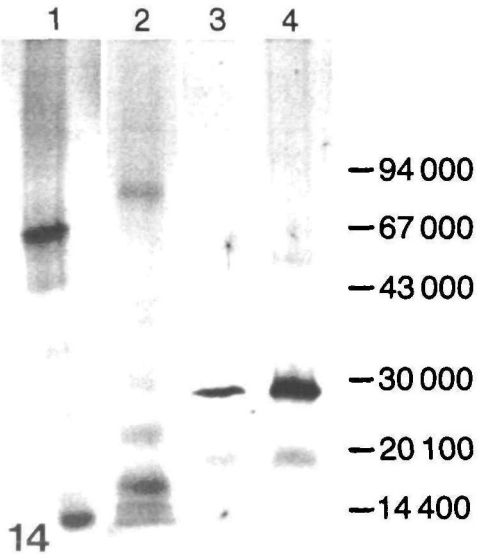

Fig. 14. SDS polyacrylamide gel electrophoresis of the cytoplasm of two gland-cell bodies (1 and 3) and of saliva collected from the proboscis lumen (2) and proboscis tip (4). The tip secretion contains only two major protein bands, and these are not found as major bands in lumen secretions. One of the cells (3) contains two protein bands which appear to correspond to those in tip secretions. Such cells could be identified in living glands by their size (approx. 600 μm) and their poor staining with Methylene Blue. The other cell (1) contains different bands, some of which correspond to those in lumen secretions.

protein analysis. Also, cytoplasm was removed from a selection of gland-cell bodies for analysis.

Gel electrophoresis showed the presence of two main protein bands in material from the proboscis tip, whereas secretions in the lumen contained at least 10 bands (Fig. 14). The tip proteins were not found in the lumen, and their relative

molecular masses (19 500 and 28 000) did not correspond to that of the anticoagulant, hementin (120 000), which is present in gland extracts (Malinconico *et al.* 1984). Interestingly, the cytoplasm of certain gland-cell bodies showed these same two bands (Fig. 14) whereas other cells had many bands, some of which corresponded to those found in lumen secretions but none to those in tip secretions (Fig. 14). More than 50 cell bodies in three leeches were examined to determine whether it was possible to identify visually the cells containing the two proteins corresponding to those found in tip secretions. Middle-sized cells (approx. 600 μm in 15 cm long leeches), which stained poorly with Methylene Blue, were consistently found to contain these two proteins, whereas other neighbouring cells did not (Methylene Blue was used because it was found to stain gland cells differentially, and this might have reflected their different secretory content). These cells were scattered throughout the gland and did not appear distributed in any particular pattern. The findings with Methylene Blue are important because there is currently no other means of distinguishing between cell types in a living gland.

Discussion

Gland-cell action potentials and secretion

The salivary gland cells of *Haementeria* resemble nerve cells in that depolarization of the cell body produces action potentials which are conducted along a process (which may be several centimetres long) to its ending where release of secretory product occurs. It is tempting, therefore, to regard the action potential as the trigger for secretion, perhaps providing an influx of calcium for exocytosis. Action potentials appear to elicit secretion in other exocrine glands such as the salivary gland and pedal gland of molluscs (Kater, 1977; Kater *et al.* 1978; Barber, 1983; Goldring *et al.* 1983) and in certain vertebrate endocrine glands (Petersen, 1980; Ozawa & Sand, 1986). Our main evidence in favour of this view is that calcium-free saline, which produces spontaneous repetitive firing in gland-cell bodies (Wuttke & Berry, 1988), also elicits copious secretion at the proboscis tip. In these experiments the glands alone were exposed to calcium-free solution by placing them in a separate compartment from the bulk of the proboscis which was bathed in normal saline.

A further link between the action potential and secretion is indicated by the fact that stimulation of the stomatogastric nerve elicits action potentials in gland cells and also causes secretion. However, secretion may also be elicited independently of action potentials, e.g. by 5-HT (see below).

Innervation of gland cells

Neuronally evoked gland-cell impulses recorded in the soma appear to be initiated outside the gland near the ductule tip and are then conducted to the cell body; they resemble the ductule spikes produced by direct stimulation of the proboscis tip. For cells with a ductule terminating at the end of the proboscis,

removal of the proboscis tip abolishes the response to nerve stimulation but not to direct stimulation of the proboscis. Our reason for examining the effect of cutting the proboscis tip was the observation by Walz *et al.* (1988) of synapses onto ductules in electron microscope sections of the proboscis tip. These authors found no evidence of synapses in the salivary gland itself (although occasional axon profiles were seen) and we have found no electrophysiological evidence of innervation of the gland. If innervation does occur only near the ductule tip then it seems unnecessary to produce propagated ductule spikes. Synapses may, however, be up to 100 μm from the ductule tip (Walz *et al.* 1988), perhaps too far to influence release directly. It is not known whether the conduction of action potentials to the cell body has any functional significance.

Application of the natural transmitter to the ductule tip would be expected to mimic the effect of nerve stimulation in producing ductule spikes and secretion. Synapses are found within 100 μm of the ductule ending at the surface of the proboscis (Walz *et al.* 1988) so that putative transmitters probably do not have to penetrate deeply into the proboscis to elicit a response. Nevertheless, saliva contains enzymes which presumably enable the penetration of mammalian skin, yet the proboscis is not obviously affected by these secretions and may well be less permeable than other leech tissues. This is indicated by the time taken for 18 mmol l^{-1} Mg^{2+} to block proboscis contractions produced by stimulating the stomatogastric nerve (20 min). Nevertheless, Glover (1987) found that, although he could detect serotonin in the proboscis and salivary glands of *Haementeria*, serotonin-immunoreactive processes were found only in the proboscis, and he suggested that the salivary glands may have a barrier to the antisera. In the present experiments two of the putative transmitters tested (serotonin and acetylcholine) produced rapid responses when applied to the proboscis tip or to the gland-cell bodies. These results suggest that there may be no special barrier in the glands or proboscis, but simply that certain parts of the tissue are less accessible than others, especially those deep in the proboscis.

5-HT: a putative transmitter

In certain jawed leeches such as *Hirudo*, 5-HT plays an important role in feeding behaviour (Lent & Dickinson, 1984) and elicits salivary cell impulses and secretion (Marshall & Lent, 1988). 5-HT also produced secretion in *Haementeria*, but without ductule spikes. Its effect on the cell body was a subthreshold depolarization and increase in excitability. Stimulation of the stomatogastric nerve did elicit ductule spikes, though a rather high frequency of stimulation was necessary to produce such spikes, and they did not occur in every cell. Dopamine was similar to 5-HT in increasing the excitability of gland cells (though less extensively) but it did not initiate secretion. Octopamine was without effect on secretion and had little effect on electrophysiology. ACh produced action potentials when applied to the cell body or ductule tip but these showed rapid adaptation and there was little or no effect on secretion. The results thus suggest only 5-HT as a transmitter candidate. The fact that it does not elicit ductule spikes indicates that the actio

potential is not the sole (or necessarily the natural) stimulus for secretion. Similarly in the pars intermedia of the vertebrate pituitary gland, secretion is produced by action potentials but can also occur in their absence, apparently as a result of spontaneous subthreshold fluctuations in membrane potential (Tomiko *et al.* 1984). In the carnivorous mollusc *Philine aperta* action potentials and excitatory synaptic potentials may independently cause release of secretion (Barber, 1983). A hormonal role for 5-HT is possible in *Haementeria*, though this was discounted in *Hirudo* by Lent & Dickinson (1984). 5-HT may possibly act as a cotransmitter because the synaptic terminals onto ductules in the proboscis tip contain small clear vesicles and large dense-core vesicles (Walz *et al.* 1988). It will be necessary to determine whether 5-HT actually occurs in the proboscis tip and whether 5-HT antagonists block the secretory response to nerve stimulation.

Additional evidence for a role of 5-HT in *Haementeria* comes from the finding by Marshall & Lent (1985) of nerve fibres with a positive reaction to anti-serotonin antibody which run along the length of the proboscis sheath and enter the salivary glands. Although it was not possible to resolve contacts with individual salivary cells, neuronal 5-HT is clearly present in their vicinity, and Marshall & Lent regard it as probably one of the natural stimuli for secretion. Glover (1987) also detected 5-HT in whole salivary glands. Our results show that there may be 5-HT receptors on the gland cell bodies but these are unlikely to have any direct role in secretion because 5-HT does not elicit action potentials. Even if the neuronal 5-HT acts to increase gland cell excitability, it is difficult to see how this could influence secretion at the distant ductule tip. There is, however, the intriguing possibility that the cell bodies are activated by a different transmitter and that 5-HT acts to ensure that repetitive firing can occur; in the absence of 5-HT the gland cells rarely produce more than three or four action potentials in response to maintained depolarization. Nerve fibres in the gland may be separate from the stomatogastric nerve and not activated by its stimulation (salivary gland cells in the mollusc, *Philine*, are innervated by independent central and peripheral neurones; Barber, 1983). The long delay between stimulation of the stomatogastric nerve and production of ductule spikes may also be indicative of indirect innervation of the ductule [though long-latency effects of transmitters are known; e.g. Gerschenfeld & Paupardin-Tritsch (1988) have described a 5-HT-induced K^+ current in snail neurones with a latency of 30–60 s and a time course of 3–5 min following a 500 ms ionophoretic application of 5-HT]. It is noteworthy that stimulation of gland-cell bodies by calcium removal elicited far more secretion than even prolonged high-frequency stimulation of the stomatogastric nerve. Any neuronal stimulation of the gland-cell bodies could thus supplement the neuronal stimulation of the ductule tip to produce maximal release of secretory products.

Secretory products

Secretions are released mainly into the lumen of the oesophagus and proboscis, and also onto the outer surface of the proboscis tip. The tip secretions were found to contain two major proteins which are probably involved with aiding penetration

of the mammalian host's skin, but the mechanism by which the soft proboscis is able to achieve this is unknown. Gel electrophoresis of extruded cytoplasm showed the same two protein bands in certain gland cell bodies. The cells could be identified in living glands on the basis of size and staining properties but extensive studies have revealed no distinguishing electrophysiological features between cells (Marshall & Lent, 1984; Wuttke & Berry, 1988). Histochemically, the gland contains three serous and two mucous secretions (Sawyer *et al.* 1982). One of the serous secretions (S1), which is probably a proteolytic polypeptide, occurs exclusively in cells whose ductules terminate at the proboscis tip (Sawyer *et al.* 1982). These cells may thus correspond with those found in the present study to release at the tip.

The anticoagulant hementin appears from histochemical studies to be released into the lumen of both the oesophagus and proboscis, where it prevents coagulation of ingested blood (Sawyer *et al.* 1982). In support of this, the present experiments showed a protein band of corresponding molecular weight in lumen secretions but not in those from the proboscis tip.

Secretory vesicles pack the cell bodies (Walz *et al.* 1988) so it is likely that secretory proteins contribute to the observed electrophoresis bands from extruded cytoplasm. In lumen secretions, bands were found that did not correspond to those in any cell, and these may represent secretions from the posterior glands which were not examined here. Some of the protein bands found in the cytoplasm did not correspond to any secretory product and probably represent nonsecretory proteins or secretory protein released in small amounts (they are perhaps associated with the nucleus which is extremely large in gland cells; Sawyer *et al.* 1982). The ability to collect cytoplasm for analysis should allow study of the metabolism of secretory products in individual cells.

Mechanism of secretion

The mechanism of release of secretions is unknown. Marshall & Lent (1985) conclude that since no acinar or duct structure is present it is likely that the various secretions are formed entirely by exocytosis without an accompanying volume of solvent, resulting in small volumes of concentrated product. Electron micrographs of the proboscis tip (Walz *et al.* 1988) add support to this view: the ductules are densely packed with secretory vesicles and the only other organelles are restricted to a narrow layer near the plasmalemma. Surprisingly, however, copious secretions can be seen to pour out of certain small, discrete areas of the proboscis tip, on occasions reminiscent of toothpaste being squeezed from a tube. It is difficult to see how this could occur by exocytosis, given the restricted access of vesicles to the ductule tip, which is only about 2 μm in diameter. One gains the impression that the tip of the ductule must have broken away (e.g. apocrine secretion). The number of sites at which this occurs is far smaller than the total number of ductule endings known to occur in the proboscis tip, so that more than one mechanism of secretion may be operating (or only a proportion of the cells are secreting). It would be interesting to look for evidence of exocytosis in electro

micrographs of the proboscis tip from an actively secreting preparation. Evidence of apocrine-type secretion could be sought by testing for the presence of vesicle membranes in the secretory product.

Single-cell model for studies of excitation–secretion coupling

The special advantages of this preparation for the study of excitation–secretion coupling are well documented (Marshall & Lent, 1984; Jones *et al.* 1985; Wuttke & Berry, 1988). The present experiments demonstrate four further important features. First, the secretions can be collected for assay, and different protein constituents resolved by gel electrophoresis. Second, the cytoplasm of individual cell bodies can be collected for analysis of secretory proteins. Third, the cells which secrete at the proboscis tip can be identified. Finally and most importantly, the secretions at the proboscis tip come from discrete points on the proboscis surface and appear to emanate from single cells. Since the salivary cells are not electrically coupled (Sawyer *et al.* 1982) this offers a unique opportunity to monitor secretion in a single, functionally isolated cell *in situ* which can be penetrated with microelectrodes.

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