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INITIATION AND MODULATION OF ACTION POTENTIALS IN SALIVARY GLAND CELLS OF *HAEMENTERIA GHILIANII* BY PUTATIVE TRANSMITTERS AND CYCLIC NUCLEOTIDES

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Summary

1. The giant salivary cells of *Haementeria ghilianii* are known to produce Ca^{2+}-dependent action potentials and to release their secretory products in response to stimulation of the stomatogastric nerve. In this study, the electrophysiological effects of some putative transmitters were examined by perfusion of the gland and two promising candidates were selected for detailed analysis.

2. Acetylcholine (ACh) was the only substance tested which excited the gland cells. It produced a large, Na^+-dependent depolarization that elicited 1–3 action potentials and desensitized to about 24 % of its maximal value within 2 min.

3. Carbachol, tetramethylammonium and nicotine elicited similar responses to ACh, whereas choline and pilocarpine had negligible effects.

4. The ACh response was completely blocked by *d*-tubocurarine and strychnine, and was reduced by tetraethylammonium, hexamethonium and atropine. The receptors, therefore, cannot be clearly distinguished as nicotinic or muscarinic.

5. ACh did not elicit secretion, but this does not necessarily preclude it from acting as a neuroglandular transmitter.

6. 5-Hydroxytryptamine (5-HT) was the only transmitter candidate that elicited secretion, though it did not excite the gland cells.

7. 5-HT produced a subthreshold depolarization and an increase in input resistance. Action potentials, elicited by depolarizing pulses, were increased in amplitude and duration, and showed greatly reduced adaptation.

8. 5-HT potentiated the net inward current, evoked by subthreshold depolarizing pulses, by reducing outward K^+ current. The inward current, carried by Ca^{2+}, was not directly affected. In addition, 5-HT increased an inwardly rectifying current, carried by Na^+ and K^+. All the effects of 5-HT tended to increase cell excitability.

9. Salivary cell responses to 5-HT were reversibly antagonised by methysergide.

10. Responses to ACh or 5-HT were not mimicked by 3′,5′-cyclic guanosine monophosphate, which greatly reduced spike amplitude and excitability. The

Key words: salivary gland, leech, *Haementeria ghilianii*, 5-hydroxytryptamine, acetylcholine, cyclic nucleotides, action potential.

effects were specific to the 3′,5′ form; 2′,3′-cyclic GMP had no effect. Cyclic GMP dramatically reduced the duration of action potentials that had been artificially prolonged by TEA^+ or removal of external Ca^{2+}.

11. Cyclic 3′,5′-adenosine monophosphate and its dibutyryl derivative had little effect on membrane properties. 8-Bromo-cyclic AMP, however, mimicked all the effects of 5-HT. It is thought that 5-HT may exert its actions *via* cyclic AMP.

12. The possible role of 5-HT in salivary secretion is discussed.

Introduction

Leech salivary cells are electrically excitable, producing calcium-dependent action potentials, accompanied by secretion, in response to nerve stimulation (Lent and Dickinson, 1984; Marshall and Lent, 1988; Wuttke *et al.* 1989). Very little is known, however, about the nature of gland-cell innervation. In the jawed leeches *Hirudo medicinalis* and *Macrobdella decora*, Marshall and Lent (1988) found that acetylcholine, γ-aminobutyric acid, dopamine and noradrenaline were without effect on secretion (though they did not examine for electrophysiological actions), whereas 5-hydroxytryptamine (5-HT) initiated secretion and excited the gland cells into impulse activity. Similar effects were produced by stimulation of the serotonergic Retzius cells, indicating that these neurones contribute to the innervation of salivary cells. The location of the synaptic input in jawed leeches is unknown. Each salivary cell extends a single process (ductule) anteriorly to the base of one of the three jaws; such ductules may reach 2 cm in length. Innervation possibly occurs close to the cell body, in view of the appearance there of subthreshold depolarizing responses to Retzius cell stimulation (Marshall and Lent, 1988).

In contrast, salivary gland cells of the proboscis-bearing leeches, *Haementeria ghilianii* and *Oosthuizobdella garoui*, are innervated near the ductule ending and action potentials are conducted back to the cell body; no signs of synaptic potentials are seen in the soma (Wuttke *et al.* 1989; Cooper and Berry, 1990). In these leeches the salivary cell bodies are collected into a distinct salivary gland whereas in *H. medicinalis* they are distributed throughout the musculature of the head. Although electrophysiological experiments failed to demonstrate any innervation of the salivary glands of *H. ghilianii*, the gland-cell bodies show responses to a number of putative transmitters (Wuttke *et al.* 1989), and axon profiles have been observed in the glands (Walz *et al.* 1988). Furthermore, Glover (1987) detected 5-HT in whole salivary glands of *H. ghilianii*, and Marshall and Lent (1985) found nerve fibres with a positive reaction to anti-serotonin antibody running along the length of the proboscis sheath and entering the salivary glands. The present paper analyses the pharmacology of the salivary gland cells of *H. ghilianii* and discusses its possible physiological significance.

Materials and methods

Preparation

Salivary glands were dissected from specimens of *Haementeria ghilianii*

(de Filippi) obtained from our breeding colony and maintained in aquaria at 26°C. The glands were secured to a layer of Sylgard at the base of a Perspex experimental bath (volume 0.25 ml) by placing pins through the overlying endothelium. Physiological saline or other solutions (see below) flowed through the bath at a rate of about 10 bath vols min^{-1}. Solutions could be changed rapidly without affecting recording conditions (Holder and Sattelle, 1972).

Observations on secretion were made with isolated preparations in which the proboscis/salivary gland complex was left intact. Unstimulated preparations produced little or no secretion, and any stimulation of secretory activity could be clearly seen at the tip of the proboscis.

Electrophysiology

Individual gland cells were impaled under visual control with two bevelled, KCl-filled microelectrodes (10–20 MΩ); one electrode was used for recording membrane potential and the other for passing either constant-current pulses to measure input resistance or direct current to alter the membrane potential. The overlying sheath was not removed or softened by enzymes. The largest cells (600–1000 μm in diameter) in the anterior salivary glands were generally chosen for study but it was not easy to maintain stable recordings with two electrodes, probably because of the greatly invaginated nature of the cell membrane (Walz *et al.* 1988); electrodes tended to come out gradually from the cell. This problem was largely overcome by mounting each electrode on a high-speed stepper (Digitimer SCAT-02), which facilitated repositioning or repenetration without loss of membrane potential. The electrodes were each connected to a Digitimer NL 102 amplifier, and signals were monitored on a storage oscilloscope (Tektronix 5111) and pen recorder (Brush 2200S) and stored on tape (Thorn EMI 3000 FM tape recorder).

Membrane currents were measured with a two-electrode voltage-clamp amplifier (Axoclamp 2A, Axon Instruments). Cells were generally held at, or close to, their resting membrane potential, and the effects of substances on the currents evoked by depolarizing or hyperpolarizing voltage steps were examined. Linear leakage currents could be subtracted electronically before displaying and storing the data.

Solutions and drugs

Physiological saline had the following composition (mmol l^{-1}): NaCl, 125; KCl, 4; $CaCl_2$, 1.8; glucose, 11; Tris maleate, 10 (pH 7.4) at room temperature (18–22°C). Ionic mechanisms were investigated by ion substitution or addition of channel blocking drugs. Na^+ was replaced by *N*-methyl-D-glucamine, and variations in $[K^+]$ were compensated by changes in $[Na^+]$. Tetraethylammonium and 4-aminopyridine (with an equimolar reduction in $[Na^+]$) were used to block K^+ currents, and Co^{2+} was used to block Ca^{2+} influx.

Putative transmitters, second messengers, agonists and antagonists were applied by perfusion at known concentrations. The following substances were used: acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, γ-aminobutyric

acid (GABA), glutamic acid (monosodium salt), dopamine hydrochloride, DL-octopamine hydrochloride, cyclic 3′,5′-adenosine monophosphate and its dibutyryl and 8-bromo derivatives (all sodium salts), cyclic 3′,5′- and cyclic 2′,3′-guanosine monophosphate (sodium salts), theophylline, methysergide maleate, carbamylcholine chloride (carbachol), pilocarpine hydrochloride, tetraethylammonium chloride (TEA^+), tetramethylammonium hydroxide, hexamethonium chloride, nicotine, strychnine, *d*-tubocurarine chloride and atropine. Apart from methysergide (Sandoz), all chemicals and drugs were obtained from Sigma Chemical Co.

Results

Acetylcholine

Of the various substances tested, acetylcholine (ACh) produced the largest effect on membrane potential. Perfusion of the salivary gland with 10^{-4} mol l^{-1} ACh produced a depolarization of 25.9±6.2 mV (N=30, mean±S.D.) accompanied by a reduction in input resistance and the generation of 1–3 action potentials (Fig. 1). This response remained unchanged with repeated applications of ACh, provided the membrane potential was allowed to return to control levels. There was desensitization with prolonged ACh perfusion, the response declining to 24±11% of its maximal value within 2 min (N=9). A concentration of 10^{-6} mol l^{-1} ACh produced a subthreshold depolarization of 6.5±2.1 mV (N=6). The minimum concentration of ACh required to produce a detectable depolarization was 10^{-7} mol l^{-1}. Acetylcholine had no effect on the amplitude and shape of action potentials.

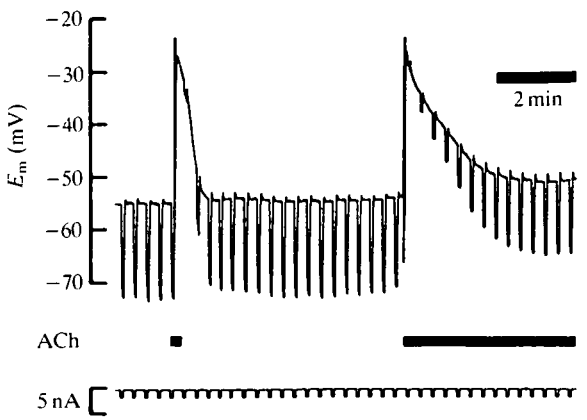

Fig. 1. Responses of a salivary gland cell to 10^{-4} mol l^{-1} acetylcholine (ACh). Two applications were made for periods indicated by the bars, and constant-current hyperpolarizing pulses, shown on the bottom trace, were injected into the cell to monitor input resistance. ACh produces a depolarization and single action potential (clipped) and an increase in membrane conductance. The response to the second application of ACh demonstrates desensitization. E_m, membrane potential.

The magnitude of the ACh response as a function of membrane potential is shown in Fig. 2 and plotted in Fig. 3. The reversal potential of the response was difficult to determine directly in most cells. Although there is no electrical coupling, which presents problems in some other salivary glands (e.g. Bahls, 1987), the large size of the cells and the extent of delayed rectification made it

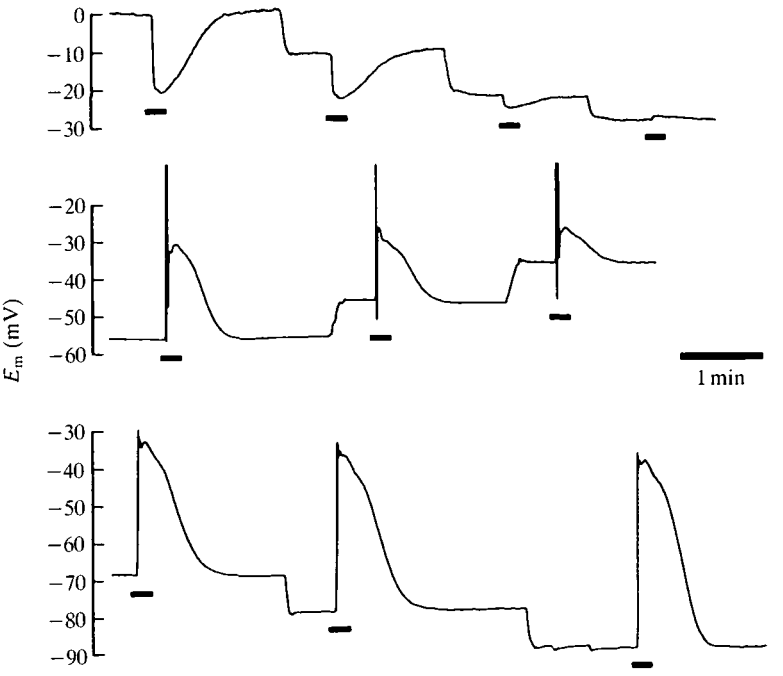

Fig. 2. Response of a salivary gland cell to applications of acetylcholine (10^{-4} mol l^{-1}, indicated by bars) at various membrane potentials. The membrane potential was adjusted by injected current.

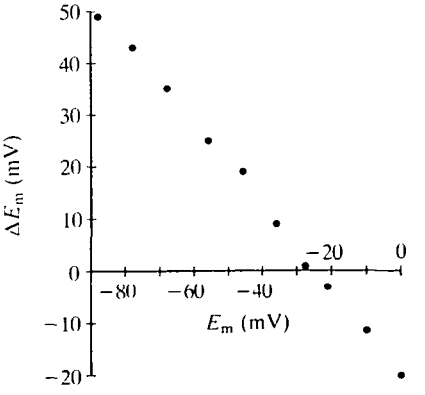

Fig. 3. Plot of the amplitude of the acetylcholine response (ΔE_m) against the membrane potential for the cell recorded in Fig. 2. The reversal potential for the acetylcholine response in this cell was −25 mV.

necessary to pass large amounts of current, which often could not be carried by the microelectrodes. In those cases where measurements were possible, the reversal potential was about −25 mV (Fig. 3).

The response to ACh was dependent on external Na^+. Replacement of Na^+ by *N*-methyl-D-glucamine produced a complete block of response in five cells, and a reduction to 18% in a further cell.

ACh agonists

Several ACh agonists were tested for their ability to mimic the response to ACh in salivary cells (Fig. 4). Each substance was tested at a concentration of 10^{-3} mol l^{-1} on 10 cells. Carbachol, tetramethylammonium and nicotine produced similar-sized depolarizations to those caused by 10^{-4} mol l^{-1} ACh, though the time course was prolonged for nicotine and carbachol. On these cells, ACh produced a depolarization of 31.2±6.3 mV, and the responses to carbachol, tetramethylammonium and nicotine were, respectively, 33.3±5.3, 33.1±4.5 and 29±5.4 mV.

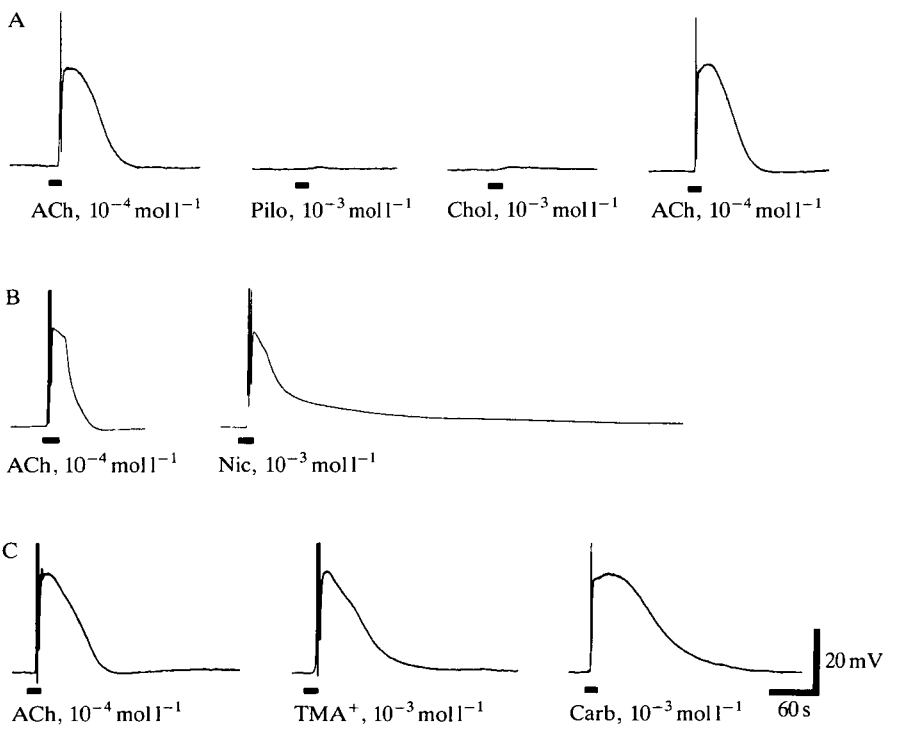

Fig. 4. Responses of salivary gland cells to acetylcholine (10^{-4} mol l^{-1}) and to various cholinergic agonists (each at 10^{-3} mol l^{-1}). The recordings in A, B and C are from three different cells in different preparations. Acetylcholine (ACh), pilocarpine (Pilo), choline (Chol), nicotine (Nic), tetramethylammonium (TMA^+) and carbachol (Carb) were perfused through the experimental bath for the period indicated by the bar below each trace.

Choline and pilocarpine (Fig. 4) produced little or no response (5.8±4.3 and 1.5±1.1 mV, respectively).

ACh antagonists

The actions of various cholinergic antagonists were examined by comparing a control response to 5×10^{-5} mol l^{-1} ACh with a test response after perfusing the gland for about 5 min with an antagonist at a concentration of 5×10^{-4} mol l^{-1}. The most powerful antagonists tested were *d*-tubocurarine (N=5; Fig. 5C) and strychnine (N=2; not illustrated), which completely blocked the response to ACh. Recovery was slow, requiring at least 25 min for *d*-tubocurarine and 40 min for strychnine. Tetraethylammonium (Fig. 5A) reduced the response to 24±5 % of control (N=4), hexamethonium (Fig. 5B) to 54±22 % (N=4) and atropine (not illustrated) to 35±18 % (N=6).

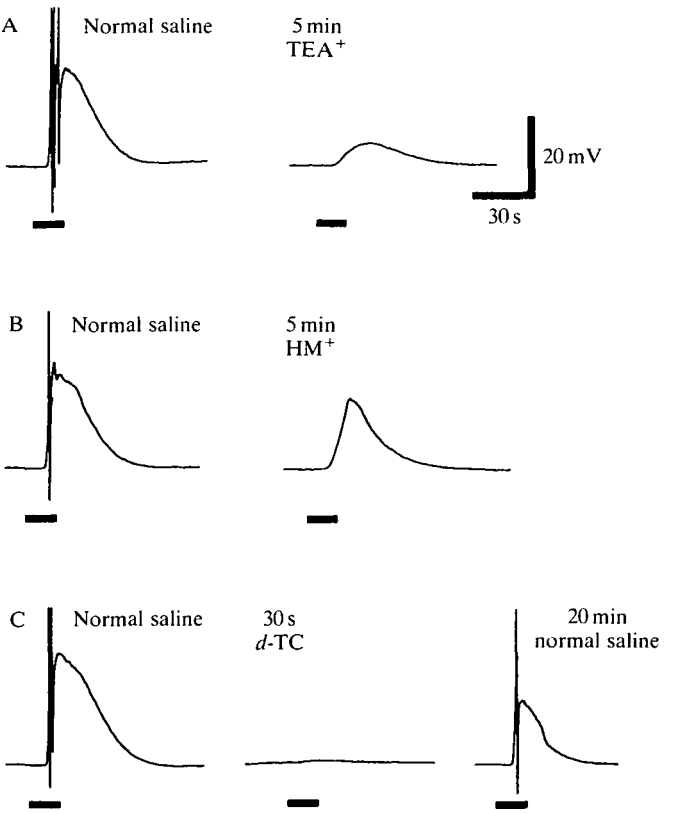

Fig. 5. Effect of cholinergic antagonists (5×10^{-4} mol l^{-1}) on the salivary gland cell response to acetylcholine (ACh, 5×10^{-5} mol l^{-1}). Recordings are from the same cell. Control responses to ACh were obtained in normal saline and following a 5 min perfusion with tetraethylammonium (TEA^+; A), hexamethonium (HM^+; B) or a 30 s perfusion with *d*-tubocurarine (*d*-TC; C). Applications of ACh are indicated by the bars below the traces. The effects were fully reversible. Recovery from *d*-TC was prolonged and can be seen to be incomplete after a 20 min wash with normal saline.

5-Hydroxytryptamine

In contrast to the large ACh-evoked responses, 5-hydroxytryptamine (5-HT) produced only a small subthreshold depolarization. Its ability to initiate secretion, however, prompted a detailed study, including voltage-clamp experiments that revealed effects on voltage-gated inward and outward currents. This paper analyses the variety of effects of 5-HT and the possible involvement of cyclic nucleotides, rather than the pharmacology of 5-HT receptors.

Membrane potential, action potential and excitability

Perfusion of the salivary glands with 5-HT produced a depolarizing response that failed to reach threshold even with concentrations as high as 10^{-4} mol l^{-1} (responses ranged from 4 to 10 mV, $N=10$). There was an associated increase in membrane resistance (18±12%, $N=9$), which was larger if the membrane potential was reset to its original value by applied current (Fig. 6). Action potentials, elicited by injection of depolarizing current, were increased in amplitude and duration by 5-HT (Fig. 6). Observable effects on these variables occurred at concentrations of about 10^{-7} mol l^{-1}, and there was no desensitization over periods of at least 15 min (longest period tested). The spike undershoot tended to be reduced by 10^{-4} mol l^{-1} 5-HT, but was little affected by lower doses.

Action potentials could be elicited by anode break at the termination of a hyperpolarizing current pulse, and these were elicited more easily in the presence of 5-HT (Fig. 6). 5-HT also enabled action potentials to be produced more readily by depolarizing pulses, and it reduced the adaptation that occurs during maintained depolarization. In the absence of 5-HT it was impossible to produce more than three or four action potentials with a depolarizing pulse, and usually only one was produced, whereas in the presence of 5-HT a train of 35 impulses or more could be elicited.

Membrane current

The effects of 5-HT were examined further by voltage-clamp studies. Since 5-HT does not initiate action potentials, but modifies their properties, emphasis was placed on the analysis of voltage-gated conductances. It proved difficult, however, to pass sufficient current through the microelectrodes to clamp cells adequately at potentials beyond threshold. Penetration with low-resistance electrodes was impossible without loss of membrane potential and excitability (this extreme sensitivity may be a general feature of leech salivary cells; for example, Marshall and Lent (1988) could not usually hold *H. medicinalis* cells (up to 200 μm in diameter) for more than 3 min with a single microelectrode). We therefore generally tested subthreshold depolarizations for effects of 5-HT that might underlie the increased size and duration of action potentials and the increased excitability. Fig. 7 shows that depolarizing pulses induced an inward current consisting of an initial fast transient followed by a longer-lasting component. In the presence of 10^{-5} mol l^{-1} 5-HT the current was greatly increased in amplitude

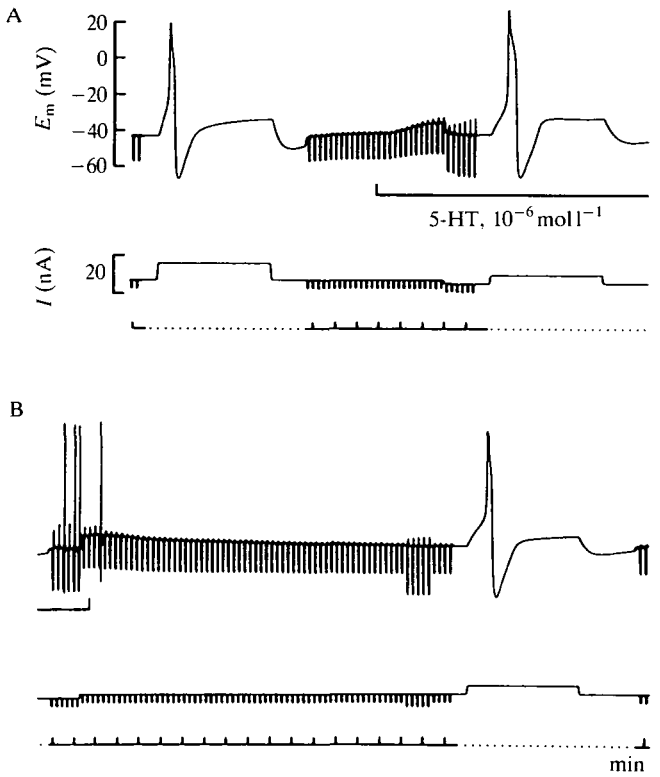

Fig. 6. Effect of 5-hydroxytryptamine (5-HT) on gland-cell activity and excitability. A and B are a continuous recording; below the traces are shown the period of 5-HT administration, the injected current (I) and a 1-min time trace. (A) 5-HT (10^{-6} mol l^{-1}) depolarized the cell and increased membrane resistance. The effect on resistance was larger when the membrane potential (E_m) was reset by applied current. Note the subthreshold after-depolarizations following each hyperpolarizing pulse in the presence of 5-HT (rebound or anode-break responses). The action potentials were elicited by depolarizing pulses. Recording speed was increased 100 times (indicated by dots) to show the increase in action potential duration produced by 5-HT. There is also an increase in spike amplitude. (B) The rebound depolarizations increase in size and begin to trigger action potentials. On washout of 5-HT the effects are reversed. Towards the end of the recording, the amplitude of the current pulses was increased to produce hyperpolarizations of similar size to those in the presence of 5-HT. Note that the rebound responses were smaller, indicating lower excitability.

recovery to initial levels followed washing with normal saline. The threshold concentration for this effect was about 10^{-7} mol l^{-1}. These and the following experiments were all performed on at least four cells and typical results are shown.

Since the cells produce purely Ca^{2+}-dependent action potentials (Wuttke and Berry, 1988), it seemed likely that the inward current was carried by Ca^{2+}, and attempts were made to distinguish between a potentiating effect of 5-HT on this current and a blocking action on K^+-mediated outward currents. No outward

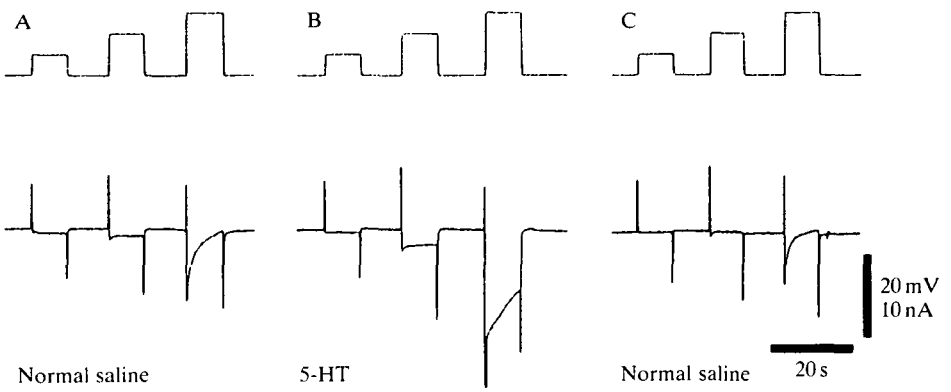

Fig. 7. Effect of 5-HT on the inward current evoked by subthreshold depolarizing pulses. (A) The membrane potential was stepped to three different levels (upper trace) while membrane current was recorded (lower trace). The third pulse, of 30 mV, induced a two-phase inward current, consisting of an initial fast transient followed by a longer-lasting component. (B) In the presence of 5-HT (10^{-5} mol l^{-1}) both components of the inward current are increased in amplitude (the initial response is clipped). (C) Recovery after washing out the 5-HT. Between pulses the cell was held at its resting level of −67 mV. At this amplification, 5-HT produced no measurable effect on the holding current. Leakage currents were subtracted.

current is apparent in Fig. 7 but, with larger depolarizing pulses or in cells with lower membrane potentials, the inward current was followed by a long-lasting outward current (see Fig. 8). Addition of 10 mmol l^{-1} $CoCl_2$ blocked the voltage-gated inward current, indicating that it is indeed mediated by Ca^{2+}. $CoCl_2$ also reduced the outward current, suggesting the presence of a Ca^{2+}-activated K^+ conductance. The effect of 5-HT was now to reduce or abolish the outward current (Fig. 8). Similar effects were produced by 5 mmol l^{-1} $CoCl_2$, except that the transient component of the inward current was not fully blocked by Co^{2+} and it was still potentiated by 5-HT. This may indicate that there are two types of Ca^{2+} channel, differing in rate of inactivation and sensitivity to Co^{2+}, but this was not investigated further.

Although the depression of outward current by 5-HT would enhance the net inward current, this did not exclude the possibility of an additional, potentiating effect on the voltage-gated Ca^{2+} current. To test this possibility we attempted to block K^+ currents by addition of 50 mmol l^{-1} TEA^+ and 10 mmol l^{-1} 4-aminopyridine. Outward currents were greatly reduced, and the inward current now showed little response to 5-HT (Fig. 9). These results suggest that 5-HT enhances inward current by reducing outward K^+ currents.

5-HT also influenced an inward current induced by hyperpolarization. Fig. 10 shows that after a hyperpolarizing jump, in addition to the instantaneous current change (linear leakage current was not subtracted), a large voltage-dependent inward current developed slowly and reached a peak within 10–20 s (depending on

voltage). Its amplitude was approximately doubled by 10^{-5} mol l^{-1} 5-HT (Fig. 10; the threshold concentration for an effect was 10^{-7} mol l^{-1}). This inward rectifier is not specific to K^+, but carries approximately equal K^+ and Na^+ currents (Wuttke and Berry, 1991).

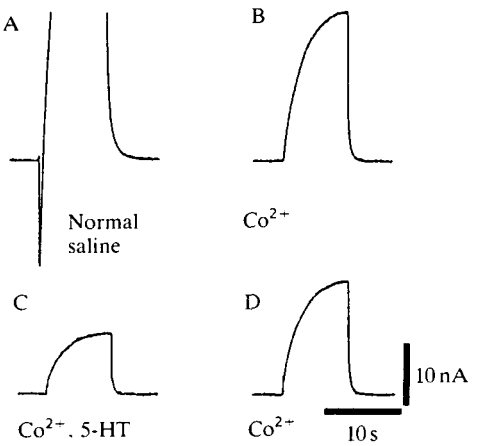

Fig. 8. 5-HT increases the net inward current, induced by depolarizing pulses, by reducing outward current. In each recording the currents were evoked by a 30 mV depolarizing pulse from −40 mV. (A) In normal saline a brief inward current is followed by a sustained outward current whose peak is off the scale. (B) In the presence of 10 mmol l^{-1} Co^{2+} the inward current is abolished and the outward current is reduced by 56%. (C) 5-HT (10^{-5} mol l^{-1}) reduces the outward current in Co^{2+} saline by 50%; there is no appearance of an inward current. (D) Partial recovery of the outward current, 10 min after washing out 5-HT. Linear leakage currents were subtracted.

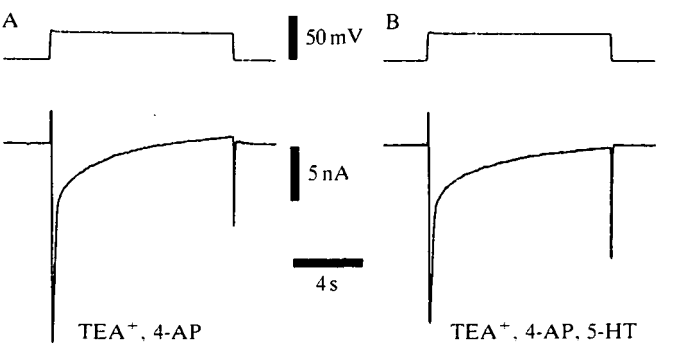

Fig. 9. 5-HT fails to potentiate the inward current, evoked by depolarizing pulses, when outward current is blocked by tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP). (A) Control current in the presence of 50 mmol l^{-1} TEA^+ and 10 mmol l^{-1} 4-AP. The membrane potential was stepped by 30 mV from −40 mV. (B) Addition of 10^{-5} mol l^{-1} 5-HT had little effect on the current (compare Fig. 7). The small linear leakage current was not substracted.

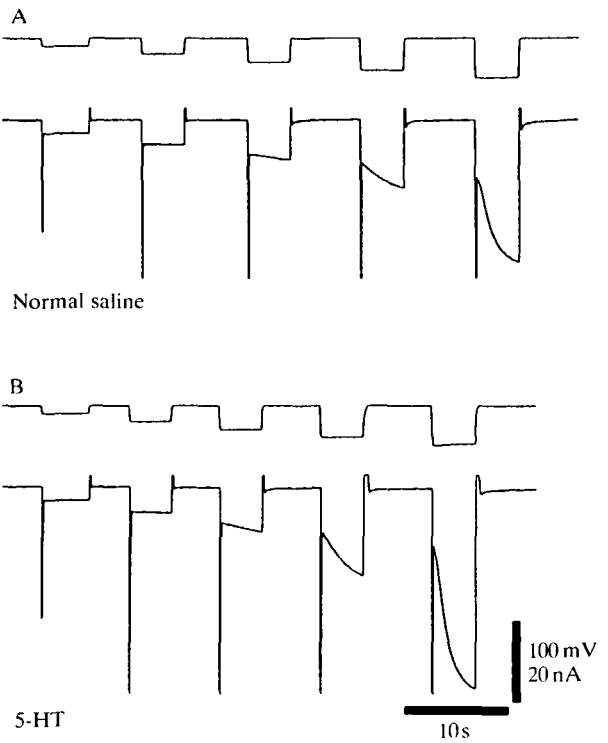

Fig. 10. 5-HT potentiates the inward current evoked by hyperpolarizing pulses. (A) A voltage- and time-dependent inward relaxation of membrane current occurs in response to 4-s hyperpolarizing voltage commands from a holding potential of −52 mV (resting membrane potential). (B) The currents are approximately doubled by 5-HT (10^{-5} mol l^{-1}). Leakage currents were not subtracted.

Effects of methysergide

A detailed analysis was not made of the pharmacological profile of 5-HT responses, but the 5-HT antagonist methysergide was used to provide information on the specificity of the actions of 5-HT. Methysergide (10^{-4} mol l^{-1}) blocked the depolarization, the increase in membrane resistance and the increased excitability produced by 10^{-5} mol l^{-1} 5-HT. The potentiating effect of 10^{-5} mol l^{-1} 5-HT on the inward current, elicited by depolarizing or hyperpolarizing steps, was reduced by 10^{-4} mol l^{-1} methysergide. All the effects of the blocking drug were reversed by washing with normal saline.

Other putative transmitters

Dopamine and octopamine produced subthreshold depolarizations, and in some cells enhanced excitability, confirming the results of an earlier study (Wuttke *et al.* 1989). Two additional substances were tested in the present study: glutamate (10^{-4} mol l^{-1}) produced a small depolarization (4.4±1.1 mV) and reduction in

membrane resistance (to 62±10%, N=7), and GABA (10^{-4} mol l^{-1}) had no effect at all (not shown). Since the electrophysiological effects of these substances were small, and none of them stimulated salivary secretion, they were not analysed further.

Cyclic nucleotides

Electrophysiological effects of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and cyclic 3′,5′-guanosine monophosphate (cyclic GMP) were examined to determine whether these substances might mediate the actions of any neuroglandular transmitter. Particular attention was paid to comparing the effects of cyclic nucleotides with those of 5-HT and ACh, which produced the major responses.

Cyclic GMP

Perfusion of the salivary glands with 1 mmol l^{-1} cyclic GMP produced a small depolarization (3.6±3.2 mV, N=9) and reduction in membrane resistance (to 54±19%, N=5), and a progressive decline in spike amplitude until the cells became very difficult to excite (Fig. 11). These effects of cyclic GMP were specific to the 3′,5′ form; 2′,3′-cyclic GMP had little or no effect. The results indicate that the actions of 5-HT and ACh are not mediated *via* this cyclic nucleotide.

There was little effect of cyclic GMP on spike duration; an initial slight shortening was followed by a small lengthening of the action potential as the amplitude fell. There was, however, a dramatic shortening of the duration of action potentials that had been artificially prolonged either by addition of 50 mmol l^{-1} TEA^+ (which produced action potentials of about 6 s duration) or by removal of external Ca^{2+} (which results in Na^+-dependent action potentials lasting tens of seconds; Wuttke and Berry, 1988). For example, the duration of Na^+-dependent action potentials was reduced by more than 90% within 1 min (Fig. 12). The speed of the response, compared with the gradual reduction in spike amplitude in normal saline, may indicate an extracellular effect. Subsequently, there was a decline in spike height, but this was associated with a progressive depolarization (Fig. 12). These experiments were not pursued further, but they clearly show complex modulatory effects of cyclic GMP on gland-cell action potentials.

Cyclic AMP

Addition of 1 mmol l^{-1} cyclic AMP or the membrane-permeable dibutyryl cyclic AMP generally produced little effect. The non-hydrolysable 8-bromo derivative of cyclic AMP (0.5–1 mmol l^{-1}), however, mimicked all the effects of 5-HT on membrane potential, membrane resistance, action potential duration and adaptation (Fig. 13) and on membrane current evoked both by depolarizing voltage steps (Fig. 14) and by hyperpolarizing steps (not shown). The effects were slowly reversible, requiring at least 30 min for full recovery. The possibility that cyclic AMP could mediate the effects of 5-HT was further tested by application of the

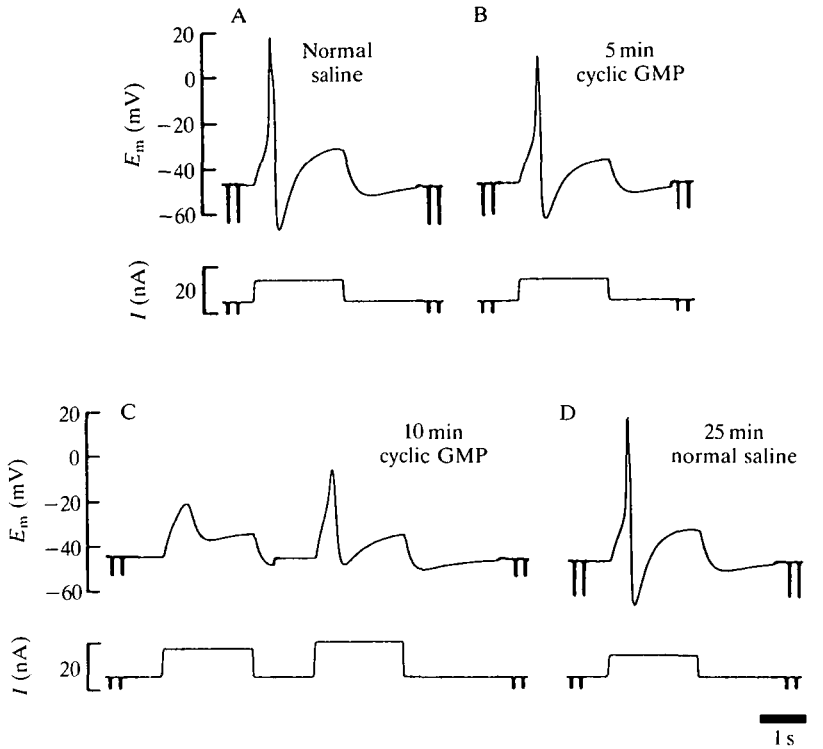

Fig. 11. Cyclic 3′,5′-guanosine monophosphate (cyclic GMP) decreases spike amplitude. (A) Control action potential (upper trace) evoked by a depolarizing pulse (lower trace). Hyperpolarizing pulses were applied to determine membrane resistance. (B) After perfusion of cyclic GMP (1 mmol l^{-1}) for 5 min the action potential is reduced in amplitude and there is a fall in resistance. (C) After 10 min the action potential fails (the membrane exhibits delayed rectification) but a larger pulse is able to elicit a small action potential. (D) Recovery after washing out cyclic GMP for 25 min. At the beginning and end of each trace, during the application of the hyperpolarizing pulses, the chart speed was slowed 100 times. I, current; E_m, membrane potential.

phosphodiesterase inhibitor theophylline (2 mmol l^{-1}). Unfortunately, no clear-cut results were obtained; any effect of theophylline was certainly not large.

Discussion

Salivary secretion in *H. ghilianii* is elicited by stimulation of the stomatogastric nerve, but attempts to discover the neuroglandular transmitter are complicated by the fact that innervation appears to occur outside the salivary gland near the ductule terminal, which is inaccessible to microelectrodes (Wuttke *et al.* 1989). Axon profiles and 5-HT-containing nerve fibres have been described in the gland (Marshall and Lent, 1985; Walz *et al.* 1988), but there is no electrophysiological or

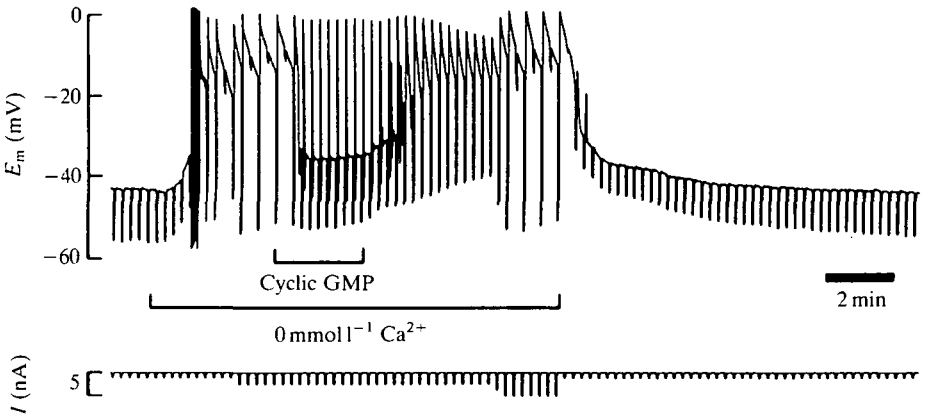

Fig. 12. Cyclic GMP reduces the duration of Na^+-dependent action potentials elicited by removal of external Ca^{2+}. In zero-Ca^{2+} saline (indicated under recording) the cell shows its usual depolarization and high-frequency burst of action potentials followed by the production of prolonged spikes. Although such spikes tend to be produced spontaneously, they were elicited as anode-break responses to hyperpolarizing pulses (bottom trace) in order to standardise their production. They can be seen to follow every other pulse immediately after the pulse amplitude had first been increased to an appropriate level. Within 1 min of addition of cyclic GMP, the spikes become greatly reduced in duration (and now follow every pulse). After washout of cyclic GMP, the spikes start to lengthen again within 1 min (they are initiated and also terminated by the pulses). There is a slow depolarization and a reduction in spike amplitude which is reversed by application of stronger hyperpolarizing pulses; this depolarization is not seen in normal saline (cf. Fig. 11).

anatomical evidence of synaptic connections there. Effects on the cell body, studied here, are therefore almost certainly extrasynaptic. In the nervous system, extrasynaptic receptors generally have no known function but may show similarities to subsynaptic receptors, and thus offer clues to the nature of any actions at the synapse (e.g. in the leech nervous system; Sargent *et al.* 1977). We have devoted most attention to 5-HT because it was the only putative transmitter tested that initiated secretion and it seems a likely candidate for the neuroglandular transmitter in *H. ghilianii* (Wuttke *et al.* 1989). Acetylcholine was also studied in some detail because it was the only putative transmitter to excite the gland cells into impulse activity.

Acetylcholine

Application of acetylcholine (ACh) to the salivary gland elicits a depolarization in gland-cell bodies that is abolished by removal of external Na^+. The reversal potential for the ACh response was found to be approximately −25 mV, which is considerably more negative than the Na^+ equilibrium potential (approx. +45 mV; Wuttke and Berry, 1990), and suggests that K^+ permeability is also increased, though this was not tested.

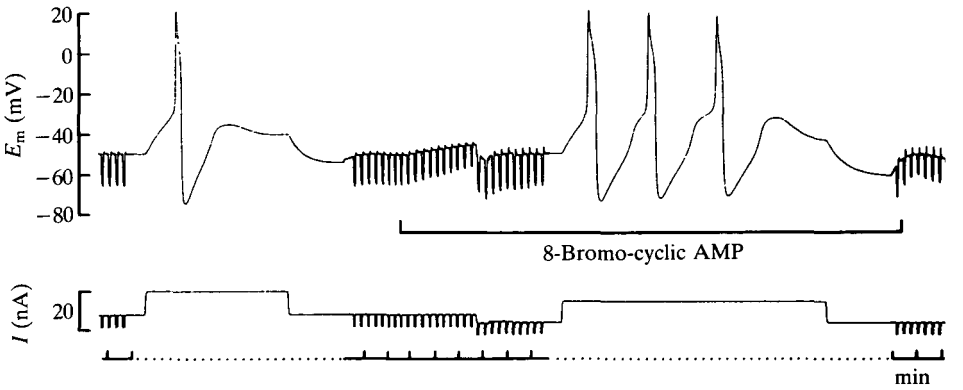

Fig. 13. 8-Bromo-cyclic AMP (1 mmol l^{-1}) mimics the effects of 5-HT on gland-cell membrane potential, resistance, impulse duration and adaptation. Application of the cyclic nucleotide results in a depolarization and increase in resistance (in this case seen only when the membrane potential is reset by applied current). A depolarizing pulse elicits a single action potential in the absence of the drug whereas three action potentials, of increased duration, are produced in its presence. The second current pulse was reduced in amplitude to allow for the increased membrane resistance and thus produce a similar-sized voltage pulse to the first. During the period indicated by the dots the chart speed was increased 100 times to allow comparison of action potential durations. E_m, membrane potential; I, current.

The ACh receptors on the salivary cells of *H. ghilianii* show similarities to those described in the salivary cells of molluscs (Barber, 1982, 1985; Bahls, 1987). Apart from annelids, only gastropod molluscs have been shown to have electrically excitable salivary gland cells, though their cellular organisation is quite different; molluscan salivary glands have an acinar structure and cell–cell coupling, typical features of all salivary glands that have been examined except for those of leeches (and probably oligochaetes; Marshall and Lent, 1988). In the mollusc *Helisoma*

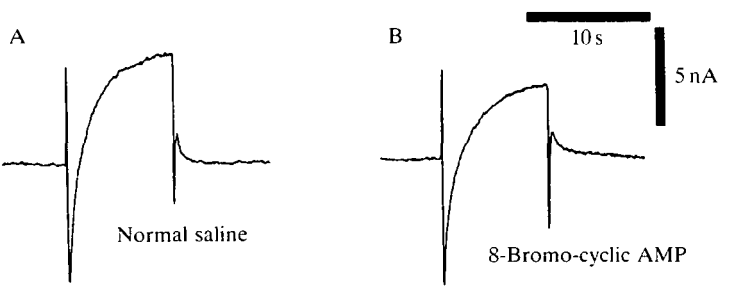

Fig. 14. 8-Bromo-cyclic AMP (0.5 mmol l^{-1}) produces similar effects to 5-HT on the currents evoked by subthreshold depolarization. (A) Control current produced by a 20 mV depolarizing step from −50 mV. (B) Exposure to 8-bromo-cyclic AMP produces a small increase in the amplitude and duration of the inward current and reduces the subsequent outward current. Leakage currents were subtracted.

trivolvis ACh produces a Na^+- (and probably K^+-) dependent depolarization that is mimicked by the cholinergic agonists carbachol and tetramethylammonium, with a weak response to pilocarpine (Bahls, 1987). Choline is, however, a potent agonist, and nicotine produces only a weak response, in contrast to their effects in *H. ghilianii*, though choline activates salivary cells in the proboscis leech *Oosthuizobdella garoui* (Cooper and Berry, 1990). The effects of cholinergic antagonists are also similar in *H. ghilianii* and *H. trivolvis*: atropine, TEA^+, hexamethonium, strychnine and *d*-tubocurarine all reduce ACh depolarizations, with the last two being the most potent and the effect of strychnine being difficult to reverse. The lack of selectivity in their sensitivity to cholinergic antagonists makes classification of the receptors as muscarinic or nicotinic difficult.

One of the main differences between the ACh response in *H. ghilianii* and *H. trivolvis* is that the molluscan salivary cells show only minimal desensitization to prolonged application of ACh and there is an after-hyperpolarization generated by the Na^+ pump (Bahls, 1987). No after-hyperpolarization is seen in *H. ghilianii*, in spite of the presence of a strongly electrogenic Na^+ pump (Wuttke and Berry, 1988), suggesting a relatively small influx of Na^+ (perhaps as a result of desensitization).

Apart from *H. ghilianii* and *O. garoui*, no detailed pharmacological studies appear to have been made of leech salivary cells. Although the salivary glands in these two species are similar in structure and physiology, some differences are apparent. For example, ACh (10^{-4} mol l^{-1}) does not affect spike amplitude in *H. ghilianii* whereas concentrations as low as 10^{-7} mol l^{-1} produce a large increase in spike height in *O. garoui* (Cooper and Berry, 1990). More marked differences are seen for 5-HT (see below).

It is possible that ACh receptors similar to those found in the cell body may occur near the ductule ending, in the region of the synaptic input. For example, application of ACh to the proboscis tip produces the same response as application to the soma, i.e. 1–3 action potentials (conducted along the ductule to the cell body; Wuttke *et al.* 1989). The failure of bath-applied ACh to elicit secretion could be due to rapid desensitization, as seen in the soma, and does not necessarily preclude ACh from consideration as a neuroglandular transmitter. Also, the rapid adaptation shown by the cells restricts their activation by bath-applied ACh. Brief applications from a micropipette might lessen the effects of desensitization and adaptation, and more closely resemble the effect of any cholinergic input. It would not be easy, however, to use this method at the ductule terminal because these cannot yet be precisely located for any one cell body.

5-Hydroxytryptamine

Although there is no direct evidence, 5-HT is a good candidate for effecting neuroglandular transmission in *H. ghilianii* (Wuttke *et al.* 1989). Better evidence is available for the jawed leech, *H. medicinalis*, where application of 5-HT or stimulation of the serotonergic Retzius cells in the first segmental ganglion evokes depolarization and action potentials in the salivary cells (Marshall and Lent, 1988).

In *H. ghilianii*, cobalt back-filling failed to demonstrate any processes of the Retzius cells in the stomatogastric nerve, which is the only source of innervation of the gland cells from the central nervous system (Wuttke *et al.* 1989). Stimulation of the Retzius cells did not evoke impulse activity in the gland cells (W. A. Wuttke and M. S. Berry, unpublished observation), though this was perhaps not unexpected since 5-HT itself does not activate the cells. This contrasts with the salivary cells of *O. garoui*, where 5-HT does not depolarize the cell body but does initiate action potentials, apparently at, or near, the ductule terminal where innervation is believed to occur (Cooper and Berry, 1990).

Electrophysiological effects of 5-HT

H. ghilianii is unusual in that 5-HT elicits salivary secretion but does not activate the salivary cells. This substance does, however, produce a multitude of effects on the cells: a depolarization and increase in input resistance, an increase in the amplitude and duration of action potentials, an increase in excitability, a reduction of adaptation during maintained depolarization, an increase in net inward current evoked by subthreshold depolarization, and an increase in an inwardly rectifying current. The relatively low concentration of 5-HT required to produce these effects (approx. 10^{-7} mol l^{-1}) and their reduction or blockade by the 5-HT antagonist methysergide indicate that they are mediated *via* specific 5-HT receptors, though no attempt was made to perform a pharmacological classification.

The depolarizing response to 5-HT is probably caused by a reduction in K^+ conductance, because it is associated with an increase in input resistance. 5-HT reduces the outward K^+ current induced by subthreshold depolarizations, thus potentiating the net inward current. These effects on K^+ conductance probably underlie the changes in excitability of the gland cells and the changed configuration of the action potentials and their rate of adaptation. The K^+ current was greatly reduced by Co^{2+}, suggesting the presence of a Ca^{2+}-activated K^+ conductance. A direct effect of Co^{2+} on the K^+ current is possible (Pollock *et al.* 1985), but substitution of Ca^{2+} with Ba^{2+} or Sr^{2+}, or removal of external Ca^{2+} without substitution by divalent cations, produced an increase in spike duration (Wuttke and Berry, 1988), again indicating a reduction in K^+ conductance.

5-HT and cyclic nucleotides

In other systems, 5-HT may mediate its synaptic effects, including those on voltage-dependent channels, *via* cyclic nucleotides (Gerschenfeld *et al.* 1986). In *H. ghilianii* the slow onset of the responses to 5-HT compared with those to ACh (cf. Figs 2 and 6) suggested the presence of a second messenger. Cyclic GMP was ruled out because of its opposing effects on input resistance, excitability and amplitude of action potentials. Cyclic AMP and its membrane-permeable dibutyryl derivative failed to produce any marked effect, but 8-bromo-cyclic AMP mimicked all the effects of 5-HT. We cannot be certain that the effects of 8-bromo-cyclic AMP represent the physiological actions of cyclic AMP, though this

phosphodiesterase-resistant nucleotide mimics the effects of cyclic AMP in many other cells (e.g. Levitan and Levitan, 1988). In the bag cells of *Aplysia californica*, where there is good evidence that 5-HT exerts its actions through cyclic AMP, both cyclic AMP and dibutyryl cyclic AMP failed to elicit a response, whereas 8-substituted derivatives produced responses similar to those of 5-HT (Kaczmarek *et al.* 1978). In *H. ghilianii*, experiments with theophylline did not provide supportive evidence, and more data are obviously required.

In *H. medicinalis* salivary cells, Marshall and Lent (1988) consider that the Ca^{2+}-dependent action potentials trigger secretion, with Ca^{2+} acting as the coupling agent. In *H. ghilianii* also, Ca^{2+}-dependent action potentials elicit secretion, but they are evidently not the only stimulus: 5-HT is a non-excitatory secretagogue, presumably acting directly on the release site at the ductule terminal (Wuttke *et al.* 1989). If 5-HT acts *via* Ca^{2+} influx, it is difficult to see how sufficient Ca^{2+} could enter during the small 5-HT-evoked depolarization (unless this is bigger at the terminal than that seen in the cell body). The present results suggest that cyclic AMP may be the coupling agent for 5-HT.

Possible modulatory role of 5-HT

Sawyer *et al.* (1982) noted a similarity between the salivary glands of *H. ghilianii* and neural ganglia. Such comparisons were shown by Marshall and Lent (1984) to be misleading because the cells are clearly not neuronal and their processes are not part of an integrative neuropile, but are totally independent of one another. Nevertheless, the individual salivary cells do bear many resemblances to neurones; for example, they extend a long process (10 cm or more) that conducts action potentials to the terminal where release of secretory products occurs, apparently as a result of Ca^{2+} influx (Wuttke *et al.* 1989). The variety of transmitter-activated or modulated conductances demonstrated here and the electrophysiological effects of cyclic nucleotides increase the similarity. The various effects of 5-HT on the salivary cells have all been found in neurones: e.g. the depolarization resulting from a decrease in resting K^+ conductance (Siegelbaum *et al.* 1982), the decrease in subthreshold voltage-gated K^+ current, with a net increase in inward Ca^{2+} current (Paupardin-Tritsch *et al.* 1981), the increase in amplitude and duration of action potentials (Gerschenfeld *et al.* 1986), the reduction in spike frequency adaptation (Andrade and Nicoll, 1987), and the potentiation of inwardly rectifying current (Benson and Levitan, 1983). Such effects allow transmitters not only to excite or inhibit but also to influence the pattern of action potential discharge, to control the way in which neurones integrate incoming synaptic input and to modulate transmitter release. Subtle effects are unlikely to be important in the salivary cells of *H. ghilianii*, which appear to remain inactive until feeding occurs and then simply pour out copious secretions. Nevertheless, several features suggest the possibility of a modulatory role for 5-HT, which does not excite the gland cells. It would be well placed to exert such effects if it is released from the synapses onto the gland-cell terminals observed under the electron microscope (Walz *et al.* 1988). This innervation is

reminiscent of input onto presynaptic terminals, for example where 5-HT has facilitatory effects on transmitter release *via* cyclic AMP (Kandel and Schwartz, 1982). Similarly, in the gland cells, 5-HT might increase release of secretory products by its effects on the amplitude and duration of the action potentials that invade the terminal. Certainly the action potential is a stimulus for secretion (Wuttke *et al.* 1989), though it is not known whether it is the natural stimulus.

Although the effects of 5-HT on the cell body are almost certainly direct, the possibility exists that 5-HT elicits secretion indirectly *via* an effect on the nervous system. Repetitive stimulation of the stomatogastric nerve (leading from the brain to the proboscis) elicits gland-cell action potentials and secretion, although it is possible that the effects are mediated by a nerve plexus in the proboscis (see Wuttke *et al.* 1989). Depending on the source of the innervation, 5-HT may thus stimulate plexus neurones or, perhaps, initiate or modulate the release of transmitter from the presynaptic terminals at the ductule ending. It would be difficult to test for such indirect effects because most procedures designed to alter synaptic transmission would also be expected to change secretion. If 5-HT does act by stimulation of the nervous system, however, it does not mimic the effect of electrical stimulation, which elicits gland-cell action potentials.

An obvious feature of the 5-HT effects is that they all tend to increase cell excitability. This includes the effect on the inward rectifier in the few cells with a high membrane potential where the rectifier is activated, but could include many more cells if their resting membrane potential *in vivo* is a little higher than that recorded in isolated glands. One possibility is that 5-HT may raise the normally low level of excitability and allow a second, excitatory transmitter to produce a maintained discharge during feeding. This is purely speculative, but the possibility that two transmitters are used is suggested by the presence of small clear vesicles and large dense-cored vesicles in synaptic terminals onto ductules at the proboscis tip (Walz *et al.* 1988).

Dual role for 5-HT

The situation is thus complex. 5-HT does not excite the gland cells and appears, from its electrophysiological effects, to be suited to a modulatory role. Nevertheless, it stimulates secretion, so perhaps a dual role should be considered. In this respect it is noteworthy that, even at high concentrations, 5-HT elicits relatively little secretion compared with amounts that can be elicited in other ways, for example by activating the gland cells with Ca^{2+}-free saline (Wuttke *et al.* 1989) or by procedures that increase intracellular pH (W. A. Wuttke and M. S. Berry, unpublished observation). Certainly 5-HT alone is not a potent secretagogue, leaving scope for additional, modulatory actions.

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