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NERVOUS CONTROL OF THE SALIVARY GLANDS OF THE CARNIVOROUS MOLLUSC *PHILINE APERTA*

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SUMMARY

Evidence is presented to show that the electrical activity of the salivary glands of *Philine aperta* is controlled by two largely independent systems which elicit different responses from salivary acinar cells. The excitatory junction potentials (EJPs) recorded from salivary cells result from the activity of a pair of identified buccal ganglion neurones. Each of these salivary effector neurones innervates only the ipsilateral gland. The effector neurones are driven to fire by synaptic input which is timed to occur during the retraction phase of the feeding cycle. Gland cell excitatory post-synaptic potentials (EPSPs) and action potentials appear to be mediated by a small group of peripheral neurones located at the base of each salivary gland. These cells give rise to a tract of fibres which cross to the contralateral gland and which may be responsible for communicating EPSP/action potential activity between the glands. The possible functions of the EJP and EPSP/spiking activities are discussed.

INTRODUCTION

The salivary glands of gastropod and cephalopod molluscs have been the subject of a number of neurochemical (e.g. Erspamer, 1952), toxicological (e.g. Ghiretti, 1960), ultrastructural (e.g. Boer, Wendelaar Bonga & van Rooyen, 1967), enzymatic (e.g. Morashita, 1974) and histochemical (e.g. Arluison & Ducros, 1976) investigations. More recently, electrophysiological studies have been made in gastropods of the nervous control of the musculature of the salivary duct (Prior & Gelperin, 1977) and the electrical activity of secretory cells (Kater, 1974, 1977; Kater, Murphy & Rued, 1978*b*; Bahls, Kater & Joyner, 1980). The regeneration of damaged neurones which project to the glands has also been studied (e.g. Murphy & Kater, 1978).

Because this previous neurobiological work was largely limited to considering the glands of herbivorous pulmonates, such as the freshwater snail *Helisoma* and terrestrial slug *Limax*, a study was undertaken to examine the nervous control of the salivary glands of a gastropod species which has a contrasting way of life. *Philine aperta* is a carnivorous marine opisthobranch mollusc whose salivary glands have already been used for studies on the electrical responses of gland cells to putative

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neurotransmitters (Barber, 1982*b*,*c*, 1983*a*). This paper describes the elements controlling the electrical activity of cells of the salivary glands of *Philine*.

MATERIALS AND METHODS

Mature specimens of *Philine aperta* were purchased from commercial suppliers at monthly intervals and kept in dark tanks which contained aerated sea water. The experiments reported below were carried out during the period April 1980–September 1982 on more than 300 animals.

All experiments were performed on semi-intact preparations which were dissected in the following way. First the buccal region was exposed by making a mid-ventral incision in the foot. Then the anterior feeding apparatus, including the anterior central nervous system (CNS), was removed by sectioning the retractor muscles, oral tube, oesophagus near the gizzard, and all cerebral and pedal nerves to the body wall (Hurst, 1965). This preparation was then incubated for 10 min in 0·1 % pronase (Type V; Sigma), after which it was pinned flat to the Sylgard-covered base of a small Perspex chamber and maintained in circulating sea water at a temperature which varied seasonally from 10–18 °C.

Intracellular recordings were made from neurones and salivary gland cells using microelectrodes filled with 3 M potassium acetate, of 50–70 MΩ resistance. Standard electrophysiological amplification, display, stimulation and recording apparatus was used.

Chemical synaptic transmission was blocked by channelling high-Mg^{2+}/low-Ca^{2+} saline (4× sea water Mg^{2+} and 0·5× sea water Ca^{2+}) through the experimental chamber. This saline was made by mixing equal volumes of sea water and isotonic (0·36 M) $MgCl_2$. During pharmacological experiments the antagonists atropine (Sigma), hexamethonium Br (Sigma), picrotoxin (Sigma) or strychnine (Sigma) were also run through the chamber. High-Ca^{2+} saline (2·5× Ca^{2+} and 2·5× Mg^{2+}), constituted as described by Getting (1981), was used to test for monosynaptic interactions.

To stain nerves with osmium the preparation was first pinned out in 9 ml of 4 % phosphate buffered formaldehyde (pH 7·4) to which 1 ml of 0·1 % OsO_4 in distilled water had been added. The specimen was then kept in the dark for 2–3 days, after which the osmium solution was replaced by sea water and the preparation was photographed under a Wild M5 microscope. Toluidene blue staining was performed using the method described by Bagust, Fitzsimons & Kerkut (1979). Cobalt backfilling and the intracellular injection of Lucifer Yellow dye were achieved using procedures previously described (Barber, 1983*b*).

RESULTS

Anatomy of the salivary system

The general anatomy of the feeding apparatus of *Philine aperta* has most recently been examined by Fretter (1939) and Hurst (1965). These authors have described the salivary system as consisting of a symmetrical pair of glands, one of which is situated on each side of the buccal cavity. The glands are unattached to the buccal region

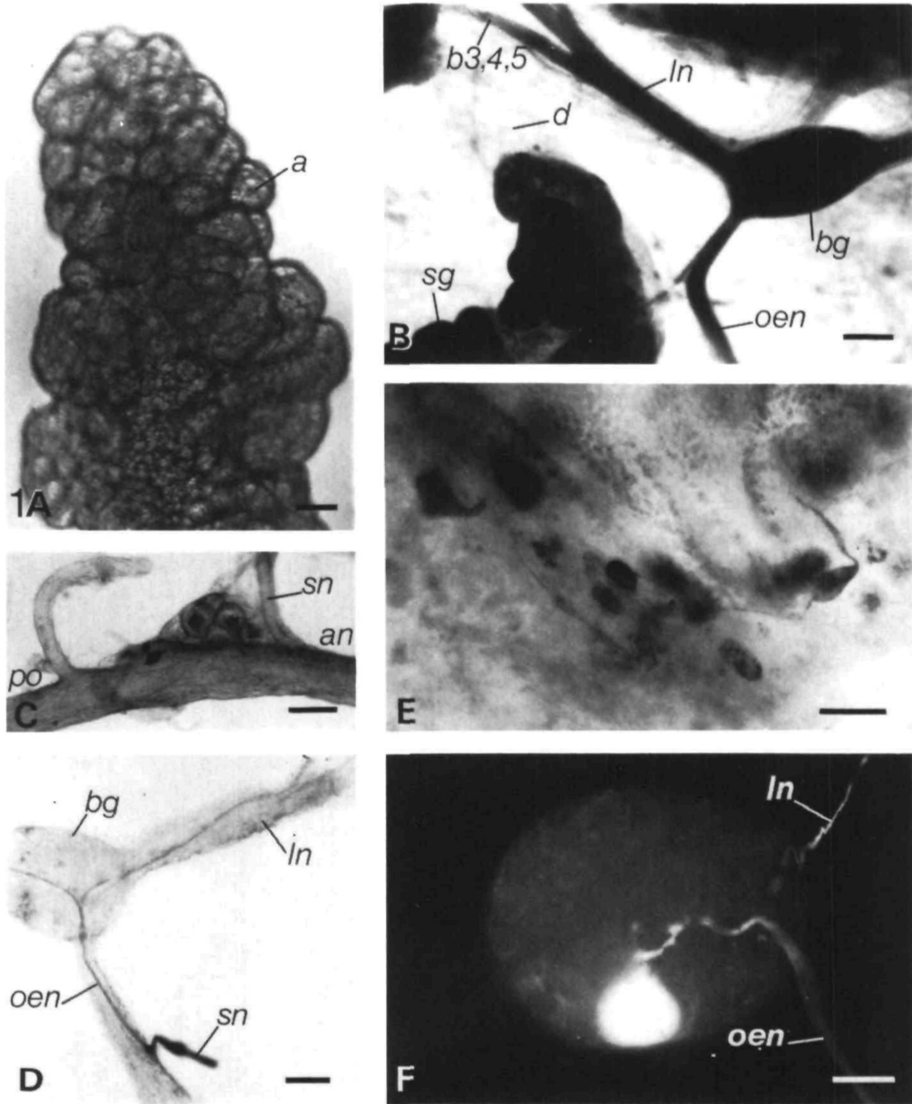

Fig. 1. (A) A living *Philine* salivary gland photographed in sea water. Acini (*a*) and individual salivary cells are visible. (B) The innervation of the salivary gland as revealed by osmium staining. A single nerve leaves the oesophageal nerve (*oen*) for the glands (*sg*). Another nerve leaves *b3, 4, 5* to innervate the salivary duct (*d*). (C) A group of peripheral neurones located in the *oen* at the base of the salivary nerve (*sn*) using toluidene blue stain. *an* = anterior, *po* = posterior. (D) A cobalt backfill of the salivary nerve showing the axons of the fibre tract. (E) Peripheral neurones at the base of the salivary gland. These neurones were stained by backfilling the salivary nerve with cobalt in the direction of the salivary gland. (F) The morphology of the salivary gland effector neurone as revealed by the intracellular injection of Lucifer Yellow dye. When stimulated, this cell elicited one-for-one EJPs in cells of the ipsilateral salivary gland. The size of the effector neurone soma is exaggerated in this photomicrograph because it is out of focus. Other abbreviations are, *bg* = buccal ganglion, *ln* = lateral nerve. Calibrations bars: A, B, D = 100 μm; C, E, F = 50 μm.

except by their ducts, and are not normally joined to one another. Salivary glands are usually between 2 and 4 mm long, are yellow-orange in colour and have the acinar structure typical of many invertebrate and vertebrate exocrine glands (Fig. 1A). Fretter (1939) has also examined the histological staining properties of the different types of secretory cells which make up the glands.

The most complete description of the nerve supply of the buccal region has been provided by Hurst (1965), who noted that the salivary glands are innervated by a branch of the oesophageal nerve. A single oesophageal nerve leaves each buccal ganglion and travels in a posterior direction, meandering over the surface of the oesophagus to the gizzard. Hurst's (1965) account also makes reference to a small ganglionic swelling, the gastro-oesophageal ganglion, which is found posterior to the point where the main branches of the oesophageal nerve leave for the dorso-lateral wall of the oesophagus.

In the present study the use of the osmium nerve staining technique confirmed Hurst's (1965) scheme for the innervation of the salivary glands (Fig. 1B). The salivary nerves were generally between 10 and 20 μm in diameter and 20 to 250 μm in length. In addition to this oesophageal salivary nerve, however, a fine nerve was observed to leave buccal nerve *b3*, *4*, *5*, crossing the surface of the oesophagus to the region of the salivary duct. In some preparations this nerve directly innervated the gland (Fig. 1B), but in others it passed around the base of the gland. Osmium treatment also revealed a network of fine nerve fibres covering the surface of the oesophagus, similar to that found in *Anisodoris* by Gorman & Mirolli (1969).

Toluidene blue clearly stained the nucleus of the large cell body in the gastro-oesophageal ganglion and also demonstrated the presence of a previously unidentified group of soma in the oesophageal nerve (Fig. 1C). This peripheral ganglion was usually located near the base of the salivary nerve and consisted of a cluster of from 5 to 15 transparent cell bodies which had diameters of between 10 and 30 μm.

Spontaneous electrical activity of gland cells

A resting potential of around −64 mV was recorded from salivary cells impaled with intracellular microelectrodes (Barber, 1982*b*). Acinar cells in isolated glands were usually electrically silent, but a variety of types of spontaneously occurring electrical activity were observed in the salivary cells of innervated glands. The intracellular injection of Lucifer Yellow through the recording electrode confirmed that this activity was recorded from secretory cells (see Barber, 1982*c*).

The most prominent type of activity was the gland action potential (Fig. 2A). These potentials were similar to those in the salivary glands of several other species of gastropod mollusc (Kater, 1977; Kater, Rued & Murphy, 1978*a*; Kater *et al.* 1978*b*; Stewart, 1981), with a long duration (up to 1500 ms) and little or no undershoot. One difference was that an action potential overshoot was never observed, whereas one is often observed in other molluscan salivary glands.

Normal spiking activity consisted of bursts of several (from 1 to 12) action potentials separated by irregular intervals, averaging between 5 s and 3 min in different animals. In some glands the mean frequency of occurrence of action potentials remained stable for several hours, but in others the ageing of the preparation was associated with a decrease in the frequency and amplitude of the salivary cell spikes.

Simultaneous recordings from cells at different ends of a single gland revealed that action potential activity was coordinated throughout each gland (Fig. 2B). Salivary cells inside each acinus showed a particularly close correspondence in their electrical

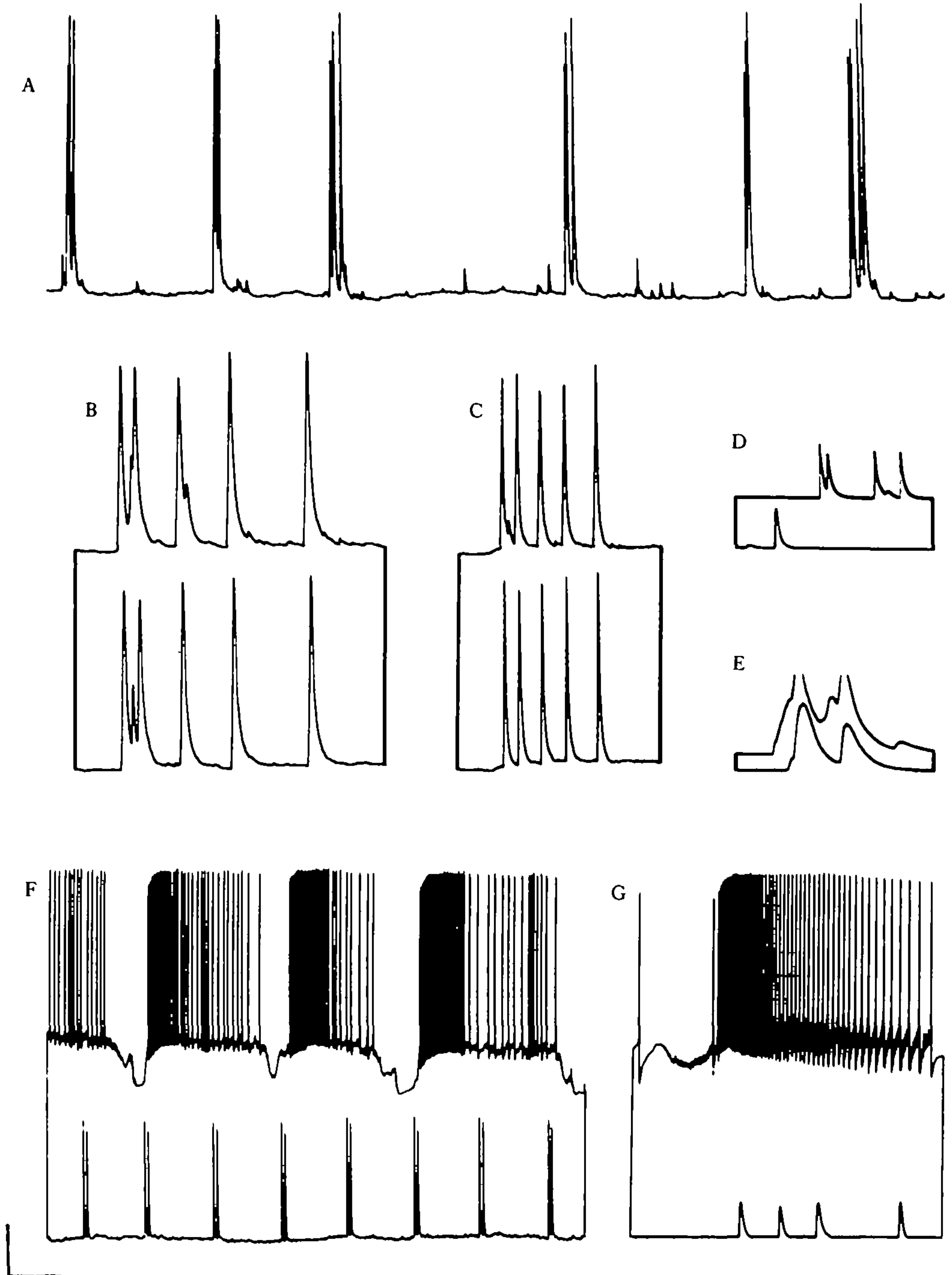

Fig. 2

activity. This spread of action potential activity throughout each gland is probably greatly assisted by the electrical coupling found between neighbouring salivary cells (Barber, 1982*c*), as in the snail *Helisoma* (Kater *et al.* 1978*a*).

By simultaneously recording the electrical activity of acinar cells in each gland it was found that action potentials also occur with a high degree of synchrony in both glands (Fig. 2C). This coincidence of action potentials was disrupted by sectioning either the buccal commissure, the oesophageal or the salivary nerves, which suggests that the coordination of spiking activity between the glands takes place through the buccal ganglia (Fig. 2D).

Another class of electrical activity consisted of small depolarizing potentials which were apparently produced by the spontaneous release of transmitter from nerve terminals on the gland (A. Barber, in preparation). These miniature synaptic potentials were distinguished from other types of gland electrical activity because they had a small and variable size, a random rate of appearance, a purely local distribution (usually confined to a single acinus), and they also showed a tendency to increase in frequency and amplitude following action potential activity (Fig. 3B; Barber, 1982*a*).

Two further categories of spontaneous activity were only recognized as being distinct when more was learned of the nervous elements controlling the salivary glands (see later). First there were the excitatory postsynaptic potentials (EPSPs) which were sometimes seen at the start of the rising phase of action potentials (Figs 2E, 3A). It seems reasonable to assume that, as in other molluscs (Kater, 1977; Kater *et al.* 1978*a*; Stewart, 1981), there are cells in the salivary glands capable of generating action potentials in response to EPSP activity. The EPSPs, like the action potentials, occurred in synchrony both within and between each gland.

Excitatory junction potentials (EJPs), similar to those produced in the non-spiking median columellar muscle of *Philine* by identified cerebral ganglion motorneurones (A. Barber, unpublished results), were also observed (Fig. 2G). EPSPs and action potentials were recorded from the same cells as EJPs, so EJPs do not represent action potentials of damaged cells. EJPs, which did not elicit action potentials, were recorded in a one-for-one manner throughout each gland, but independently in the right and left glands.

Another point of difference between the EPSP/action potential and EJP activities was their relationship to bouts of motor activity in the feeding apparatus. Coordinated sequences of buccal muscle movements which resembled feeding occurred spontaneously in the preparations used in this study. Sigger & Dorsett (1981) have

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Fig. 2. Spontaneous electrical activity of gland cells. (A) Gland action potentials and miniature synaptic potentials recorded from an acinar cell. (B) Action potential activity is synchronized between the proximal (lower trace) and distal (upper trace) ends of a single gland. (C) Synchronous action potential activity in left (upper trace) and right (lower trace) glands. (D) Sectioning the buccal commissure abolishes this correspondence in action potential activity. (E) EPSPs recorded from proximal (upper trace) and distal (lower trace) gland cells at the start of the rising phase of salivary action potentials. (F) The upper trace is from an unidentified R-phase neurone which is undergoing spontaneous cycles of activity, while the lower trace is from a salivary cell in the ipsilateral gland. The firing of the buccal neurone bears no relation to the occurrence of salivary gland action potentials. (G) Recording from an unidentified R-phase neurone (upper trace) and a salivary cell in the ipsilateral gland (lower trace). Bursts of gland cell EJPs are confined to the R-phase of the feeding cycle. Calibrations: (A) 7 mV, 6 s; (B) 10 mV, 1·5 s; (C) 10 mV, 3 s; (D) 40 mV, 1·5 s; (E) top 8 mV, bottom 20 mV, 650 ms; (F) 10 mV, 5 s; (G) 10 mV, 2 s.

described the bursting patterns of certain buccal ganglion neurones associated with these movements and they have classified their firing activity as occurring either during the phases of protraction (P) or retraction (R) of the buccal mass. Surprisingly, no correlation was found between this presumed feeding behaviour and the timing of the salivary action potentials (Fig. 2F). Bursts of EJPs, when they were observed, were recorded only during the R-phase of the feeding cycle (Fig. 2G).

Gland cell responses to nerve stimulation

Spikes, and potentials resembling EPSPs and EJPs, were readily elicited from the acinar cells of isolated glands by stimulating the cut end of the ipsilateral salivary nerve. Control stimuli, which were applied when the salivary nerve had been displaced from the stimulating suction electrode, elicited no gland cell response even at the highest stimulus strengths (15 V).

The stimulation of the salivary nerve produced propagating action potentials from proximal and distal gland cells after a delay of between 10–20 ms, and up to 50 ms,

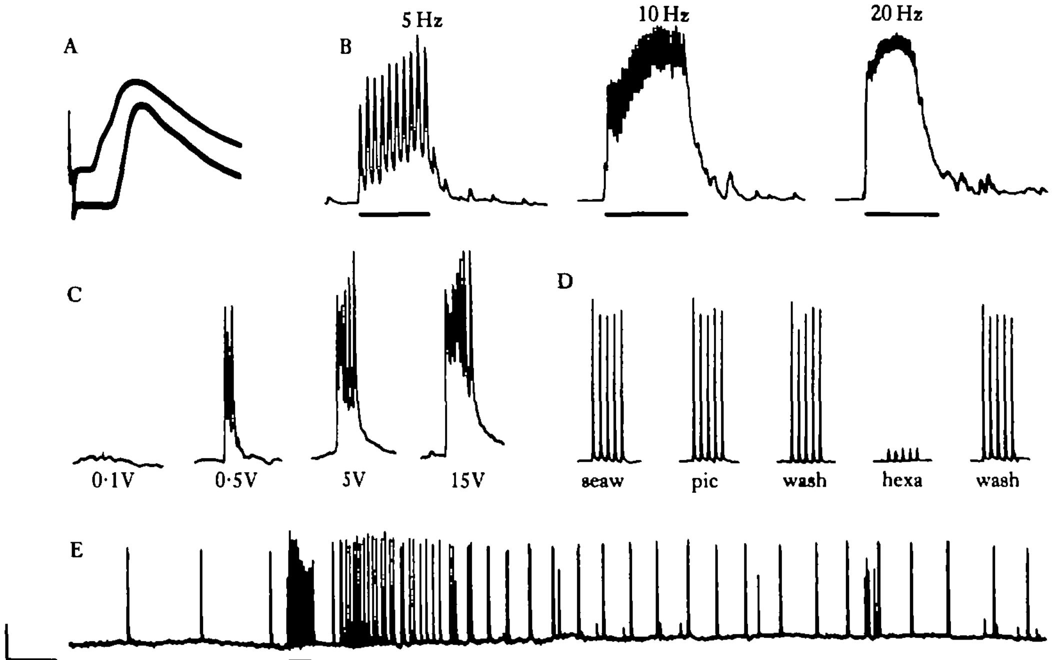

Fig. 3. Gland cell responses to nerve trunk stimulation. (A) Response of a proximal (upper trace) and a distal (lower trace) salivary cell to the stimulation of the ipsilateral salivary nerve. (B) At stimulus frequencies between 10–20 Hz the one-for-one relationship between stimuli and gland cell responses begins to break down. The solid line underneath each record indicates the stimulus duration. N.B. the increased discharge of miniature synaptic potentials after the end of stimulation. (C) Increasing the strength of a single brief (10 ms) stimulus to the ipsilateral salivary nerve generates several action potentials from a gland cell. (D) The responses of gland cells to a series of five stimuli to the salivary nerve in sea water (seaw) were unaffected by soaking the preparation for 30 min in 10^{-4} M-picrotoxin (pic). 10^{-4} M-hexamethonium (hexa) blocked the response to nerve stimulation within 2 min of being introduced to the experimental chamber. This effect was reversed by washing the preparation for 5 min in sea water. (E) A series of 15 impulses applied to the buccal commissure elicit one-for-one salivary action potentials, and also cause a delayed and prolonged increase in the frequency of spontaneous spiking activity. Calibrations: (A) 20 mV, 80 ms; (B) 20 mV, 2 s; (C) 8 mV, 4 s; (D) 10 mV, 10 s; (E) 20 mV, 20 s.

respectively (Fig. 3A). Action potentials were generally able to follow sustained trains of stimuli up to a frequency of 10 Hz (in innervated glands, natural rates of spiking as high as 8 Hz have been observed). At stimulus frequencies of around 10–20 Hz, however, this one-for-one relationship between nerve stimulation and gland response began to fail (Fig. 3B).

By increasing the intensity or duration of the stimulus to the salivary nerve it often proved possible to produce several action potentials for every stimulus (Fig. 3C); as many as 11 salivary cell action potentials have been elicited by a single brief shock (10 ms). These results presumably indicate that the salivary glands are innervated by more than one excitatory nerve fibre, each fibre having a different threshold for response to electrical stimulation.

Gland cell responses were also recorded in response to stimulation of the ipsilateral and contralateral oesophageal and lateral nerves, the buccal commissure and contralateral salivary nerve, but not buccal nerve *b1* or either cerebro-buccal connective (see Fig. 4A). Similarly, no response was observed following the stimulation of the cut end of the ipsilateral lateral nerve in the direction of the cerebral ganglion. This last result probably means that the branch of the *b3, 4, 5* nerve (Fig. 1B) innervates only the duct of the salivary gland and does not influence the activity of the gland cells.

The responses of acinar cells to the stimulation of the salivary nerve were unaffected by perfusing the experimental chamber for 30 min with either 10^{-4} M-atropine or picrotoxin (Fig. 3D). Pharmacological studies have already shown that atropine and picrotoxin antagonize the effects of externally applied serotonin or octopamine, and GABA, respectively (Barber, 1983*a*). The amine and GABA receptors, which are believed to be located presynaptic to the neuro-glandular junctions (Barber, 1982*c*, 1983*a*), do not, therefore, appear to mediate the effects of salivary nerve stimulation. 10^{-4} M-hexamethonium, which probably acts directly on postsynaptic gland cell receptors (Barber, 1983*a*), rapidly and reversibly blocked the response to nerve stimulation (Fig. 3D).

In addition to the direct effects described above, nerve stimulation also produced marked changes in the frequency of spontaneously occurring gland cell action potential activity. Stimulation of all the major ipsi- and contralateral buccal nerve trunks, including the cerebro-buccal connectives, the lateral nerves, the oesophageal nerves (both posterior and anterior to the salivary nerve) and also *b1* and the buccal commissure, caused a prolonged increase in the rate of appearance of salivary action potentials after a short delay (Fig. 3E).

These effects may be mediated, at least in part, through the axonal processes of a pair of identified serotonin-containing neurones, known as the SCNs, which are found in the cerebral ganglia (Barber, 1983*b*). The SCNs, which have axons with a wide distribution in the nerves of the buccal ganglia, are known to be able to increase the frequency of gland cell spikes when stimulated by the intracellular injection of depolarizing current (Barber, 1983*b*).

Cobalt backfills of the salivary nerve

Nerve elements with projections from the CNS to the salivary glands were stained by cobalt backfilling salivary nerves in the direction of the buccal ganglia.

The most commonly observed feature of these backfills was that of a tight bundle

of approximately 20 very fine fibres which travelled along the oesophageal nerve to the ipsilateral buccal ganglion (Figs 1D, 4A). Upon entering the ganglion the fibre tract divided into two. One group of fibres passed down the lateral nerve and buccal nerve *b3*, *4*, *5* and then left to cross the oesophagus to the base of the salivary duct. The other fibre bundle entered the buccal commissure and travelled to the contralateral ganglion, the number of stained fibres gradually diminishing as the distance from the ipsilateral salivary nerve increased. No cell bodies were ever found associated with these fibres in either buccal ganglion. In the contralateral ganglion the tract again divided. Some fibres projected to the salivary duct *via* the lateral nerve and buccal nerve *b3*, *4*, *5*. The remaining fibres passed down the contralateral oesophageal and salivary nerve, from where they innervated both the proximal and distal regions of the contralateral gland.

The cell body of a single buccal ganglion neurone was also demonstrated in salivary nerve backfills (Fig. 4B). This neurone, which was 40–50 μm in diameter, was consistently located in the mid-region of the exposed ventral surface of each ganglion. In addition to its projection to the ipsilateral salivary nerve, this cell also innervated the salivary duct *via* the lateral nerve in the same manner as the fibre tract. Unlike the processes of the fibre tract, however, the backfilled buccal cells had no axons with a contralateral destination.

The last feature found in these backfills was that of a thick axon which projected down most of the nerve trunks of the buccal ganglia (Fig. 4C). This axon probably belongs to the SCN neurone of the cerebral ganglion, which is known to have processes of a similar size in many buccal nerves, including the ipsilateral salivary nerve (Barber, 1983*b*).

One pair of cells which were not stained are the S-cells of the buccal ganglion. These S-cells are two primary mechano-sensory neurones which respond to the stimulation of the oesophageal wall and salivary glands (Dorsett & Sigger, 1981). They are known to have processes in the salivary nerves from intracellular dye injection studies (Dorsett & Sigger, 1981) and nerve trunk stimulation experiments (A. Barber, unpublished observations). It is not at present clear why the S-cells do not fill with cobalt.

By backfilling down the salivary nerve in the direction of the gland, it proved possible to trace the destinations of the fine processes of the fibre tract (Fig. 4D). After reaching the gland, the fibre bundle usually divided into two. One set of fibres travelled to the distal part of the gland, each acinus receiving a rich supply of very fine axons. Some of these fibres had features resembling dendritic swellings. The other group of fibres passed down the salivary duct to the oesophageal wall at the base of the gland where they joined to the cell bodies of a number (as many as 13) of peripheral neurones (Fig. 1E). These cell bodies were bipolar in form and had diameters ranging from 10 to 40 μm.

Thicker axons, presumably those of either the buccal neurone, the SCN or the S-cells, were only occasionally filled.

Effects of identified neurones on gland activity

Extensive searches, on both sides of the buccal ganglia of many preparations, have failed to reveal any neurone capable of generating action potentials in salivary cells

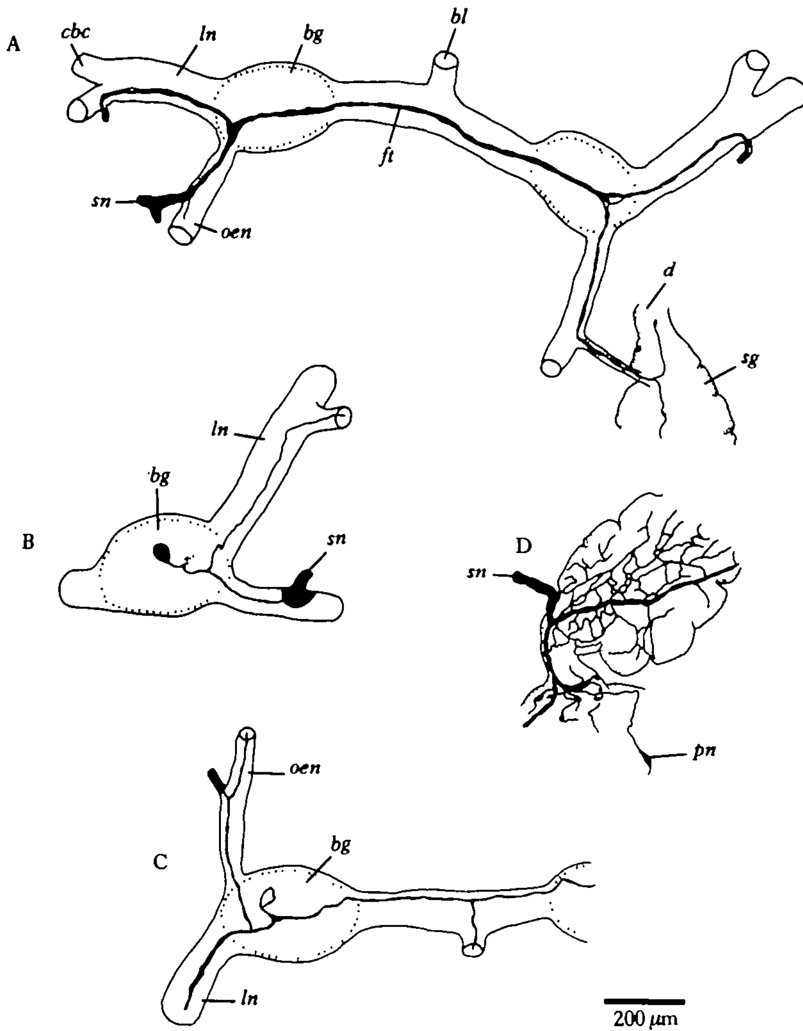

Fig. 4. Cobalt backfills of the salivary nerve. (A) Diagram showing the axonal projections of the fibre tract (*ft*) identified in salivary nerve backfills. The *ft* sends fibres to the ipsilateral salivary duct and contralateral salivary gland. (B) A single buccal ganglion neurone was sometimes revealed by nerve backfilling, as was a thick axon with extensive projections to most buccal nerve trunks (C). (D) Backfilling down the salivary nerve to the glands showed that the fibres of the *ft* originate from a group of small peripheral cell bodies at the base of each gland. Other abbreviations are *bl* = buccal nerve 1, *bg* = buccal ganglion, *cbc* = cerebro-buccal connective, *d* = salivary duct, *ln* = lateral nerve, *oen* = oesophageal nerve, *pn* = peripheral neurone, *sg* = salivary gland, *sn* = salivary nerve.

A neurone was found, though, which produced gland cell EJPs when stimulated. This undistinguished medium-sized neurone was normally located between the large cells of the O-group (see the buccal cell map of Sigger & Dorsett, 1981) in the same position as the cell body identified in the cobalt backfills of the salivary nerve (Fig. 4B). The morphology of this buccal ganglion neurone, which was revealed by the intracellular

injection of Lucifer Yellow (Fig. 1F), bore a close resemblance to that of the backfilled buccal cell (Fig. 4B).

The salivary EJPs produced by the stimulation of the buccal neurone were recorded from acinar cells throughout the ipsilateral, but not contralateral salivary gland after a delay of around 30 ms (Fig. 5A). This EJP activity was not associated with any mechanical movement of the salivary gland. Evoked-EJPs often had large amplitudes (up to 25 mV) and usually showed no signs of either summation or facilitation. Not all salivary cells in the ipsilateral gland received EJPs from the buccal neurone, however. It is estimated that only about one in five salivary cells in the ipsilateral gland show a detectable postsynaptic response to the stimulation of the buccal ganglion cell. This may be one reason why the neurone is often rather difficult to find.

EJPs proved capable of following spikes in the buccal cells one-for-one at high frequencies with a constant latency. Further evidence of a monosynaptic connection between the buccal neurone and cells of the salivary gland was obtained by soaking the preparation in high-Ca^{2+} saline. Because the threshold for spike generation is raised in high-Ca^{2+} media, polysynaptic pathways are more likely to fail since intermediate neurones are less able to produce action potentials (Berry & Pentreath, 1976). In the present study it was found that prolonged contact with high-Ca^{2+} saline did not

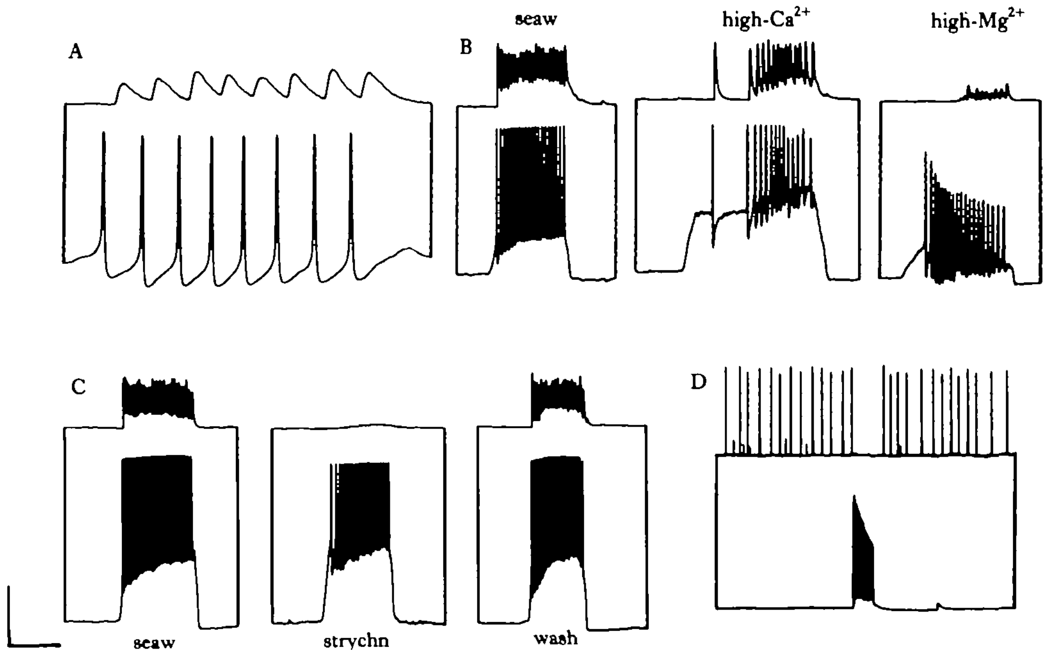

Fig. 5. Effects of identified buccal ganglion neurones on glandular activity. (A) Artificial depolarization of the salivary effector neurone produces one-for-one EJPs in salivary cells of the ipsilateral gland. (B) This one-for-one relationship between effector neurone spikes and salivary EJPs seen in sea water (seaw) persists when the preparation is bathed in high-Ca^{2+} saline. Effector neurone EJPs are much reduced in size by high-Mg^{2+}/low-Ca^{2+} saline. All these records are taken from the same preparation. (C) Effector neurone-induced EJPs are antagonized by 20 s contact with 5×10^{-4} M-strychnine (strychn). The blocking action of strychnine is reversed by washing the preparation for 10 min in sea water. (D) Spontaneous salivary gland action potential activity (upper trace) is inhibited by firing the ipsilateral S-cell (lower trace). Calibrations: (A) top 10 mV, bottom 20 mV, 100 ms; (B) top 10 mV, bottom 20 mV, 1 s; (C) top 10 mV, bottom 20 mV, 2 s; (D) top 10 mV, bottom 20 mV, 40 s.

affect the one-for-one relationship between buccal neurone spikes and salivary cell EJPs (Fig. 5B). This suggests that the buccal neurone is a salivary gland effector neurone.

EJPs produced by the stimulation of the effector neurone were reversibly abolished, or much reduced in size, by bathing the preparation in high-Mg^{2+}/low-Ca^{2+} saline (Fig. 5B). This result indicates that the effector neurone probably mediates its effect on gland cells *via* chemical synapses. The action of the effector neurone was also rapidly and reversibly blocked by the antagonists strychnine, hexamethonium and atropine. Fig. 5C shows how 5×10^{-4} M-strychnine blocked effector neurone EJPs within 20 s of being introduced into the experimental chamber; this blocking action was then reversed by washing the preparation for 10 min in sea water.

In addition to the effector neurones, the S-cells were able to influence the electrical activity of the salivary glands. Spontaneous spiking in both glands was inhibited by generating a burst of action potentials in either S-cell, though this effect was usually weak (Fig. 5D). The firing of the S-cells similarly interrupted the train of salivary cell action potentials produced by the introduction of octopamine or serotonin (Barber, 1982*c*).

No evidence was obtained to suggest that there was any relationship between the firing of the oesophageal nerve peripheral neurones (Fig. 1C) and glandular activity. Moreover the intracellular injection of Lucifer Yellow into these neurones did not reveal any axons projecting onto the salivary glands.

It was not possible to make intracellular recordings from the peripheral neurones at the base of the salivary gland (Figs 1E, 4D) because these cells were not visible in living preparations.

Effector neurone synaptic input, firing patterns and interactions with other identified neurones

The salivary gland effector neurones generally received little or no synaptic input and normally showed few spontaneous action potentials. During the occasional spontaneous cycles of buccal mass movements and contractions of the feeding muscles, however, these neurones exhibited a distinctive type of firing activity.

Simultaneous recordings from salivary effector neurones and buccal ganglion neurones active during the periods of protraction or retraction of the buccal mass (Sigger & Dorsett, 1981) revealed that bursts of effector neurone action potentials were confined to the R-phase of the 'feeding' cycle (Fig. 6A). This effector neurone spiking was driven by a burst of high-frequency excitatory synaptic input (Fig. 6B) which was shared by other, but not all, R-phase neurones (Fig. 6C). Unlike every other R-phase neurone examined, the salivary effector neurones did not receive the prominent wave of inhibition during the preceding P-phase.

Because it was difficult routinely to identify salivary effector neurones through their postsynaptic action on gland cells, in the following experiments effector neurones were located by stimulating the cut end of the ipsilateral salivary nerve and recording antidromic action potentials from the soma. When injected intracellularly with Lucifer Yellow, these cells were found to have a similar morphology and the same axonal projections as the effector neurones. The results obtained in this way were found to be in full agreement with findings from the previous experiments.

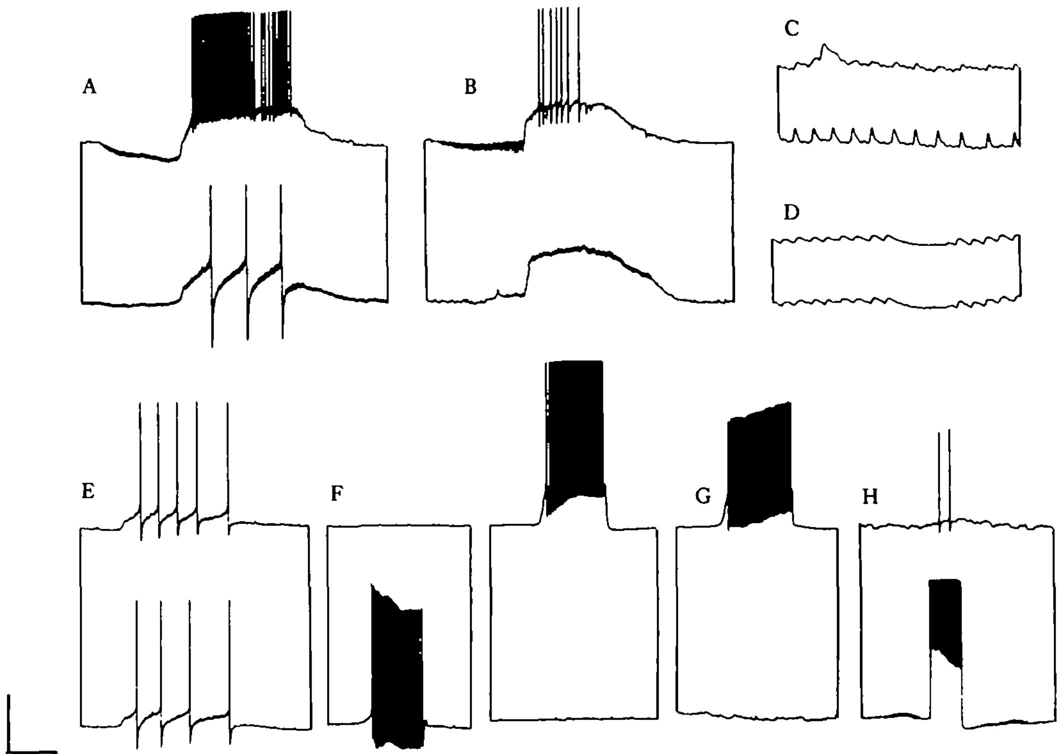

Fig. 6. Effector neurone firing patterns and interactions with identified neurones. (A) The salivary effector neurone (lower trace) fires in time with an unidentified R-phase buccal neurone (upper trace) during a cycle of feeding-like motor activity. The spikes in the salivary effector neurone trace are clipped by the pen recorder. (B) Salivary effector neurones (lower trace) receive a barrage of high-frequency synaptic input during the R-phase of the feeding cycle. The upper trace is from an unidentified R-phase neurone. (C) The R-phase synaptic input to the salivary effector neurone (lower trace) is shared by some other R-phase buccal neurones (upper trace). (D) A paired recording from both salivary effector neurones showing that the R-phase input occurs synchronously in the two neurones. (E) A simultaneous recording from both salivary effector neurones which shows that although the two neurones receive identical synaptic input during the feeding cycle, this does not result in one-for-one spiking in the two cells. (F) Artificial generation of spikes in the left salivary effector neurone (first pair of records) and the right effector neurone (second pair) reveals no evidence of interaction between these two cells. (G) The firing of the mechanosensory S-cell (upper trace) has no effect on the ipsilateral salivary effector neurone (lower trace). (H) Stimulation of the ipsilateral SCN neurone (lower trace) generates action potentials in the salivary effector neurone (top trace). Calibrations: (A) top 10 mV, bottom 5 mV, 2 s; (B) top 10 mV, bottom 5 mV, 2 s; (C) 5 mV, 100 ms; (D) 5 mV, 100 ms; (E) 10 mV, 2 s; (F) 10 mV, 2 s; (G) top 20 mV, bottom 10 mV, 2 s; (H) 10 mV, 10 s.

Although the EPSPs of the R-phase input occurred synchronously in each salivary gland effector neurone (Fig. 6D), individual synaptic potentials were not large enough to produce matching action potential activity in both cells (Fig. 6E). Effector neurone spikes appeared to result from the summed depolarization produced by the small EPSPs, each neurone firing as it reached its own threshold.

Simultaneous recordings from both salivary effector neurones did not reveal any evidence of either direct or indirect connections between these neurones (Fig. 6F). Unlike other R-phase buccal ganglion neurones (Sigger & Dorsett, 1981) the salivary

effector neurones received no detectable synaptic input from the mechanosensory β-cells (Fig. 6G).

An excitatory connection was observed between the ipsilateral and contralateral SCN neurones of the cerebral ganglia (Barber, 1983*b*) and the salivary effector neurones (Fig. 6H). The synaptic potentials underlying this excitation were not detected, however, and it is not known whether the interaction between these cells is mono- or polysynaptic.

DISCUSSION

Identified neurones and glandular activity

Although it has not proved possible to record intracellularly from the peripheral neurones located at the base of each salivary gland (Figs 1E, 4D), the evidence available suggests that these cells and their processes (i.e. the fibre tract) may mediate the EPSP and spiking activity recorded from gland cells. The results of those experiments in which the salivary nerve was stimulated indicate that each gland is probably innervated by several excitatory axons. These axons produce salivary cell action potentials when they are stimulated (Fig. 3C). The findings of the pharmacological experiments are consistent with the proposal that the gland cells are directly innervated by these nerve fibres, and are not excited indirectly through the mediation of presynaptic processes on the glands (see Barber, 1983*a*). Moreover, salivary gland spikes are generated only when the nerves which contain axons of the fibre tract are stimulated. The stimulation of nerves such as *b1*, which do not receive fibre tract axons (Fig. 4A), does not directly elicit salivary gland action potentials. The identity of the neurotransmitter which mediates salivary EPSP and action potential activity is not known.

Gland cell EJPs, on the other hand, appear to result solely from the firing of the ipsilateral salivary gland effector neurone. Cobalt backfills of the salivary nerve have revealed only one cell body in each buccal ganglion which has an axon projecting to the ipsilateral salivary gland (Fig. 4B), and the morphology of this cell closely resembles that of the effector neurone (Fig. 1F). Artificial stimulation of the effector neurone produces EJP activity in the ipsilateral gland (Fig. 5A) while the effector neurones are normally active during the R-phase of the feeding cycle (Fig. 6A), which coincides with the timing of EJP activity recorded from gland cells (Fig. 2G). Evidence from the pharmacological experiments, taken together with the results of previous work (Barber, 1982*b*), indicates that the salivary effector neurone may be cholinergic.

The EJPs evoked by stimulating the effector neurones have never been observed to trigger salivary gland action potentials, despite their direct and powerful excitatory action on gland cells and the close electrical coupling which is found between neighbouring salivary cells (Barber, 1982*c*). It seems likely, therefore, that some mechanism exists to prevent EJPs from generating spikes in those gland cells which have voltage-dependent membrane properties.

Agonist-induced uncoupling of gland cells may be important in this context. Acetylcholine (ACh) applied externally to the salivary gland has been shown to reduce the transmission of electrotonic potentials between neighbouring salivary cells, and

ACh is also able to block the propagation of action potentials throughout the gland (Barber, 1982*c*). It is not yet known, however, if this effect can be reproduced by stimulating the effector neurone.

The pair of serotonin-containing SCN neurones of the cerebral ganglia (Barber, 1983*b*) probably influence the electrical activity of the salivary glands only indirectly. The excitatory effects of externally applied serotonin (Barber, 1982*c*) on salivary cell action potential activity in isolated glands are believed to result from events which occur presynaptic to the neuroglandular junction, presumably on axons of the fibre tract.

The SCNs also influence EJP activity in the glands by lowering the threshold at which the salivary effector neurones fire (Fig. 6H). Whether or not the SCNs also act postsynaptically to modulate the size or duration of the effector neurone EJPs, or change the electrical properties of the acinar cells, is not yet known, but serotonin does not appear to influence the electrical responses of gland cells to bath-applied ACh (Barber, 1982*c*).

The weak inhibitory action of the mechanosensory S-cells on salivary gland spiking (Fig. 5D) may also be mediated presynaptically because there was no noticeable effect on the membrane potential of the gland cells when the S-cells were stimulated. Nor were hyperpolarizing potentials ever detected following the extracellular stimulation of the salivary nerve.

A summary of the proposed organization of the salivary system of *Philine* is shown in Fig. 7.

Functions of EJP and EPSP/action potential activities

Philine salivary glands appear to have two largely independent control systems: the buccal ganglion effector neurones, and the salivary gland peripheral neurones. These control systems elicit different responses from acinar cells and probably serve different functions in the working of the glands.

The effector neurone and gland cell EJP activities are clearly related to the periodic bouts of feeding-like motor activity recorded from neurones of the buccal ganglia (Figs 2G, 6A). Since EJP activity occurs only during the consummatory phase of the feeding cycle, this suggests that the salivary gland secretion induced by the EJPs may have a role during the swallowing of the prey. One possible function of this secretion is to lubricate the passage of food down the oesophagus.

The timing of the salivary gland EPSPs and action potentials, however, bears no relationship to these spontaneous cycles of buccal neurone activity (Fig. 2F). Salivary gland action potential activity is often found to continue steadily for several hours and would, therefore, seem to be intended to produce a continuous release of secretion into the buccal cavity. In *Philine* the oesophagus has replaced the stomach as the chief site of extracellular digestion (Fretter, 1939). The prey, which consists of small molluscs and worms, is swallowed whole and then thoroughly crushed between the calcareous plates of the triturating gizzard. This process can continue for many hours, during which time the food is exposed to the action of a number of digestive enzymes. The presence of an amylase and glycogenase has been demonstrated in the salivary glands and also in the fluid found in the lumen of the oesophagus (Fretter, 1939). Perhaps the salivary gland spiking activity is responsible for the discharge of enzymes into the oesophagus as part of the process of digestion.

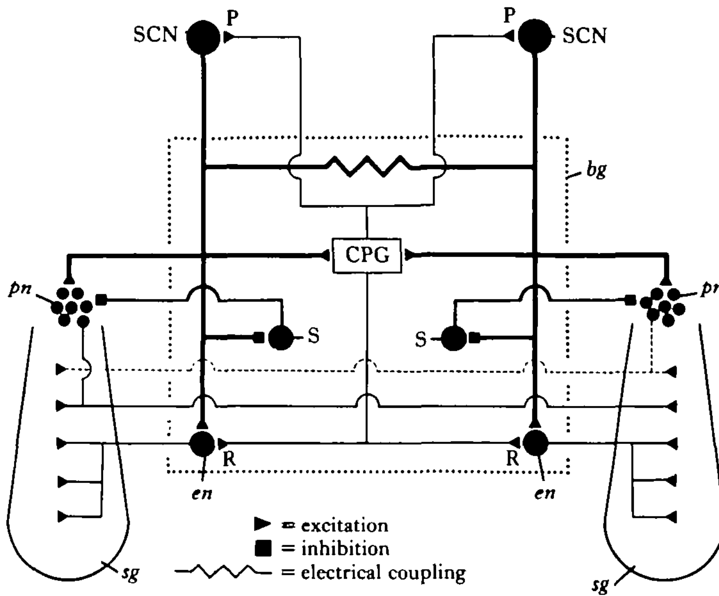

Fig. 7. Schematic diagram showing the proposed organization of the *Philine* salivary system. This diagram also incorporates data from Barber (1983*b*). Abbreviations: *bg* = buccal ganglia, CPG = hypothetical feeding central pattern generator, *en* = effector neurone, P = input during buccal mass protraction, *pn* = peripheral neurones, R = input during buccal mass retraction, S = mechanosensory S-cell, SCN = serotonergic cerebral neurone, *sg* = salivary gland. The action of the S-cell on the contralateral salivary peripheral neurones, and the action of the SCN on the contralateral S-cell and effector neurone, are not shown.

Comparisons with other glands

There has been no demonstration of a dual control apparatus incorporating both central and peripheral neurones in any other gastropod salivary system examined to date. An identified peripheral neurone in each gastro-oesophageal nerve of the opisthobranch *Anisodoris* is known to have an axonal projection to the ipsilateral salivary nerve (Gorman & Mirolli, 1969), so peripheral neurones may have a role of some kind in the control of the salivary glands of certain other gastropods.

The action of central neurones on the electrical activity of the salivary gland cells is better understood. Differences clearly exist between the salivary effector neurones in species from different groups (Table 1), but the effector neurones of the closely related pond snails *Helisoma* and *Lymnaea* are very similar, and may possibly be homologous (see also Stewart, 1981).

It is significant that the salivary effector neurones of at least three gastropod species appear to be cholinergic. There is a possibility that neuroglandular transmission in some other species may also be cholinergic, because ACh-like compounds have been detected in the salivary glands of different gastropods (e.g. Asano & Itoh, 1959, 1960; Fange, 1960).

Table 1. *Properties of some gastropod salivary effector neurones*

	Philine	*Helisoma*	*Lymnaea*	*Limax*	*Helix*
Effector neurone projections to salivary nerve	Ipsilateral	Bilateral[2, 13]	Bilateral[6]	Ipsilateral[15]	Bilateral[1]
Gland cell response to effector neurone stimulation	EJP	EPSP/action potential[2, 11]	—	—	—
Electrical coupling between effector neurones	absent	present[2, 11]	present[5]	indirectly through *b7* neurones[16]	—
Activity synchronized with buccal mass	retraction	retraction[10, 11]	protraction[5]	protraction[15]	—
Response to mechano-sensory input	none	none[10]	—	excitation[4]	—
Effect of homologous serotonin-containing cerebral neurone on the effector neurones	excitatory but no PSPs recorded	polysynaptic EPSPs[9]	monosynaptic EPSPs[12]	monosynaptic EIPSPs predominantly inhibitory[8]	monosynaptic EPSPs[7]
Suggested effector neurone transmitter	ACh	—	—	ACh[3]	ACh[14]

— Means the property has not been determined.
[1] Altrup & Speckmann (1982). [2] Bahls, Kater & Joyner (1980). [3] Barry & Gelperin (1982). [4] Beltz & Gelperin (1980). [5] Benjamin & Rose (1979). [6] Benjamin, Rose, Slade & Lacy (1979). [7] Cottrell & Macon (1974). [8] Gelperin (1980). [9] Granzow & Rowell (1981). [10] Kater (1974). [11] Kater, Murphy & Rued (1978*b*). [12] McCrohan & Benjamin (1980). [13] Murphy & Kater (1978). [14] Osborne (1977). [15] Prior & Gelperin (1977). [16] Prior & Grega (1982).

In addition to *Philine* salivary glands, there are other examples of glands capable of exhibiting two kinds of response to stimulation. Acinar cells of the opaline gland of the opisthobranch mollusc *Aplysia*, for instance, respond to motoneurone stimulation with voltage-independent EJPs, but action potentials, unassociated with the activity of these cells, can also be recorded from gland cells (Tritt & Byrne, 1980). In the mammalian pancreas, field stimulation produces a voltage-independent depolarization of exocrine gland cells (Davison & Pearson, 1979), whereas the endocrine β-cells generate action potentials in response to glucose (Dean & Matthews, 1970). These β-cell action potentials are not recorded from cells throughout the pancreas, however, so in this respect the physiology of the pancreas differs from that of the *Philine* salivary glands.

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