The Journal of Experimental Biology **199**, 407–413 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0024

THE EFFECTS OF SEROTONIN AND DOPAMINE ON SALIVARY SECRETION BY ISOLATED COCKROACH SALIVARY GLANDS

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Accepted 17 October 1995

Summary

We have studied the effects of 3-hydroxytyramine (dopamine) and 5-hydroxytryptamine (serotonin) on (1) the rates of salivation from isolated salivary glands of the cockroach *Periplaneta americana*, (2) the protein content of the saliva, and (3) the ultrastructure of the salivary gland epithelium. The rates of neurotransmitter-induced salivation varied in a dose-dependent manner within the concentration range 10^{-9} to 10^{-4} mol l^{-1}. Half-maximal secretory rates were induced by 6×10^{-7} mol l^{-1} serotonin and 1.1×10^{-7} mol l^{-1} dopamine. Stimulation of the glands by serotonin resulted in the production of a protein-rich saliva, whereas saliva was protein-free after stimulation by dopamine. Electron microscopic studies revealed that the central cells, which are believed to produce the proteinaceous components of the saliva, secrete their vesicular content after stimulation by 10^{-6} mol l^{-1} serotonin for 20 min. In contrast, no morphological changes could be detected after stimulation by 10^{-6} mol l^{-1} dopamine. These data indicate that dopamine stimulates only the secretion of the fluid component of the saliva, whereas serotonin is necessary to stimulate secretion of the proteinaceous components.

Key words: 5-hydroxytryptamine, serotonin, dopamine, secretion, salivary glands, *Periplaneta americana*, cockroach.

Introduction

Two major types of salivary glands can be distinguished in insects: tubular glands, as are found in blowflies and some lepidopterans, and the morphologically more complex acinar salivary glands of, for example, cockroaches and locusts (for a review, see House, 1980). The distal part of the tubular glands in the blowfly *Calliphora vicina* consists of a single cell type that carries out the two basic functions of saliva formation: (1) electrolyte and water transport from the haemolymph to the glandular lumen and (2) the production and secretion of proteins and mucins. These glands are not innervated, and secretion is stimulated by the neurohormone serotonin (Oschman and Berridge, 1970).

In contrast, the secretory acini of the salivary glands in cockroaches contain two morphologically distinct cell types, the peripheral cells and the central cells. Morphological characteristics indicate that the former are specialized for electrolyte/water transport, the latter for the production and secretion of proteins and mucins (Kessel and Beams, 1963; Bland and House, 1971; Just and Walz, 1994*a*). These glands are innervated from the suboesophageal ganglion and the stomatogastric nervous system (Whitehead, 1971). A number of histochemical (Bland *et al.* 1973; Elia *et al.* 1994), electrophysiological (House and Ginsborg, 1979; Ginsborg *et al.* 1974; House *et al.* 1973) and pharmacological (Evans and Green, 1990*a*,*b*, 1991) investigations indicate that dopamine functions as a neurotransmitter. However, other substances, particulary serotonin and various catecholamines, have been shown to mimic electrical nerve stimulation in electrophysiological experiments (Bowser-Riley and House, 1976; House and Smith, 1978; House and Ginsborg, 1979); they are also able to evoke the secretion of saliva (House and Smith, 1978). Thus, our understanding of which specific neurotransmitter stimulates acinar salivary glands *in vivo* is limited. In particular, no information is available on which cell type is stimulated by which neurotransmitter. Additionally, previous investigations have examined only the mechanisms of fluid secretion and, to our knowledge, the stimulating agonist for the secretion of the proteinaceous components of the saliva is unknown.

The aim of the present study was to investigate the effects of serotonin and dopamine on the secretory response of isolated cockroach salivary glands and on the protein content of the saliva produced. In addition, we have studied the morphological changes in the different cell types of the glands following stimulation by either serotonin or dopamine.

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Materials and methods

Animals

A colony of *Periplaneta americana* (L.) (Blattodea, Blattidae) was reared at 27 °C, in 70–80 % relative humidity, with a light/dark (L:D) cycle of 12 h:12 h, and with free access to food and water. Only male imagos were used.

Physiology

Measurements of fluid secretion and the collection of saliva for analytical purposes were carried out using a modification of the perfusion technique of House and Smith (1978). The paired salivary glands were dissected as described previously (Just and Walz, 1994*a*) and immediately transferred to a Ringer-filled chamber, as illustrated in Fig. 1. The chamber was perfused continuously (10 ml min^{-1}) with oxygenated cockroach physiological saline, containing (in mmol l^{-1}) 160 NaCl, 10 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 $NaHCO_3$, 1 NaH_2PO_4, 5 Tris, 4 HCl, 20 glucose, pH 7.4. The main salivary duct of one gland was separated from the adherent reservoir duct. Its free end was pulled into an oil-filled chamber (filled with liquid paraffin) and fixed with a pin. Near its fixed end, the duct was perforated to allow secreted saliva to flow into the oil, where it formed a growing droplet. The glands were stimulated by perfusion of the Ringer chamber with variable neurotransmitter concentrations (10^{-9} to 10^{-4} mol l^{-1}) of either 5-hydroxytryptamine (serotonin, Sigma, Deisenhofen, Germany) or 3-hydroxytyramine (dopamine, Sigma), diluted in cockroach physiological saline. Samples of the secreted fluid were removed every minute with gel-loader tips (Eppendorf) and transferred to a second paraffin pool. The volume of the spherical saliva droplets was calculated from their diameter.

Protein detection

Glands were stimulated with either 10^{-6} mol l^{-1} dopamine or 10^{-6} mol l^{-1} serotonin for 20 min. Samples of the secreted fluid were immediately diluted 1:2 in gel-loading buffer containing 0.125 mol l^{-1} Tris buffer pH 6.8, 2 % SDS, 0.01 % Bromophenol Blue, 17.4 % glycerine, 26 mmol l^{-1} dithiothreitol (DTT), 150 μmol l^{-1} EDTA and 0.05 % NaN_3. After centrifugation for 2 min at 12 000 revs min^{-1} (Eppendorf centrifuge 3200, Hamburg, Germany), the samples were heated in boiling water for 5 min to denature proteins, cooled to room temperature, centrifuged again and stored overnight at −20 °C. The samples were loaded on 15 % SDS–polyacrylamide gels and electrophoresis was carried out at a constant current of 30 mA. Proteins were detected by silver staining according to the protocol given by Blum *et al.* (1987, modified). Briefly, the gels were incubated for 90 min in 50 % methanol, 12 % acetic acid and 0.02 % formaldehyde and then rinsed for 10 min first in 50 % ethanol and then in 30 % ethanol. They were then incubated for 1 min in 0.8 mmol l^{-1} $Na_2S_2O_3$, rinsed with distilled water, incubated for 20 min in 0.1 % $AgNO_3$ and 0.03 % formaldehyde, rinsed again, and developed for 3 min in 6 % Na_2CO_3, 0.02 % formaldehyde and 1.6 μmol l^{-1} $Na_2S_2O_3$. Finally, the silver staining was stopped using 5 % acetic acid for 5 min, and the gels were rinsed several times in distilled water before examination.

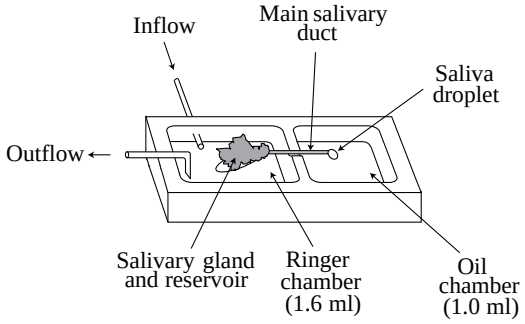

Fig. 1. Schematic representation of the perfusion chamber used for the collection of saliva from isolated cockroach salivary glands.

Electron microscopy

Salivary glands were stimulated for 5 min or for 20 min using either 10^{-6} mol l^{-1} dopamine or 10^{-6} mol l^{-1} serotonin and were immediately transferred to a fixative containing 3.5 % glutaraldehyde and 5 mmol l^{-1} $CaCl_2$, buffered with 0.1 mol l^{-1} sodium cacodylate to pH 7.4. The glands were prefixed in this solution for 1 h at 4 °C, washed with 0.1 mol l^{-1} sodium cacodylate buffer and postfixed in a mixture of 1 % OsO_4 and 0.8 % $K_3Fe(CN)_6$ in 0.1 mol l^{-1} sodium cacodylate buffer. All specimens were stained *en bloc* for 2 h with 2 % aqueous uranyl acetate, then dehydrated through a series of ethanol concentrations, and embedded in ERL 4206 (Spurr, 1969). The blocks were sectioned on an Ultracut OMU3 microtome (Reichert-Jung, Nußloch, Germany) and examined with a transmission electron microscope 10C/CR (Carl Zeiss, Oberkochen, Germany) at 60 kV.

Results

Physiology

Under optimal experimental conditions, isolated salivary glands responded to stimulation by secreting fluid for several hours. Nevertheless, stimulation experiments were carried out within approximately 1.5–2 h. After this time, run-down effects became significant. Individual preparations varied in size, in their average rate of secretion and in their tendency to run down. Therefore, a standard stimulation procedure was chosen that led to highly reproducible results (Fig. 2). (1) In order to compare the secretory responses of different preparations, one standard neurotransmitter concentration (10^{-6} mol l^{-1} dopamine) was given in every experiment and all measured secretory rates were normalized to the secretory rate evoked by stimulation with the standard concentration. (2) To exclude the influence of gland run-down on the quantification of secretory rates, the standard neurotransmitter concentration was given before and after the test concentrations. (3) In each preparation, only two different neurotransmitter concentrations were tested and the responses were normalized to the secretory rate evoked by the standard concentration. (4) Each

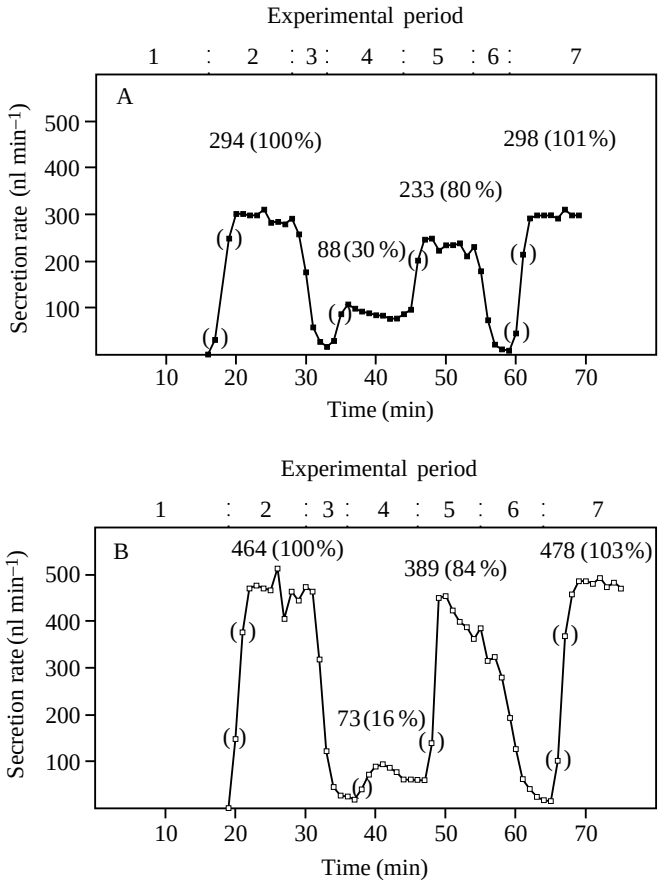

Fig. 2. Representative secretory responses of individual salivary glands to stimulation by different concentrations of neurotransmitter. Zero on the abscissa marks the beginning of the isolation procedure of the glands from the living animal. Mean volumes secreted every minute (in $nl\,min^{-1}$) are given for each period of stimulation. The average secretory response to the first application of the standard ($10^{-6}\,mol\,l^{-1}$ dopamine) was set at 100 %. (A) Dopamine stimulation experiment: period 1, cockroach physiological saline (Ringer); period 2, $10^{-6}\,mol\,l^{-1}$ dopamine (standard); period 3, Ringer; period 4, $10^{-7}\,mol\,l^{-1}$ dopamine; period 5, $2\times10^{-7}\,mol\,l^{-1}$ dopamine; period 6, Ringer; period 7, $10^{-6}\,mol\,l^{-1}$ dopamine. (B) Serotonin stimulation experiment: period 1, Ringer; period 2, $10^{-6}\,mol\,l^{-1}$ dopamine; period 3, Ringer; period 4, $2\times10^{-7}\,mol\,l^{-1}$ serotonin; period 5, $10^{-4}\,mol\,l^{-1}$ serotonin; period 6, Ringer; period 7, $10^{-6}\,mol\,l^{-1}$ dopamine. Symbols in parentheses represent sub-maximal secretory rates at the beginning of the response to neurotransmitter.

neurotransmitter concentration was applied for 10 min. The length of time between applications of neurotransmitter was sufficient for adequate recovery because the amount of secreted saliva did not change if a shorter or longer recovery time was allowed. The secreted saliva droplets were removed every minute and their volumes determined. Secretory rates ($nl\,min^{-1}$) are given as the arithmetical mean of the volumes secreted every minute.

Fig. 2 illustrates the results of two experiments following stimulation with different concentrations of either dopamine (Fig. 2A) or serotonin (Fig. 2B). The unstimulated glands showed no secretory activity. The secretory response to each transmitter concentration began within a few seconds but it took approximately 2–3 min until the gland secreted at its final dose-dependent rate (Fig. 2, points in parentheses). This behaviour was observed for both neurotransmitters. Glands recovered more slowly from serotonin stimulation than from dopamine stimulation (compare Fig. 2B, periods 3 and 6).

Under our experimental conditions, the secretory rates were approximately constant over the time of stimulation (Fig. 2A, periods 2, 4, 5, 7; Fig. 2B, periods 2 and 7). High serotonin concentrations produced secretion rates that declined slowly from an initial peak rate in some preparations (Fig. 2B, period 5). This decline did not affect the secretory rate induced by the following stimulation by the standard solution containing $10^{-6}\,mol\,l^{-1}$ dopamine (Fig. 2B, period 7).

The dose–response curves for serotonin and dopamine, calculated from experiments with 34 different preparations, are shown in Fig. 3. The average secretion rate stimulated by $10^{-6}\,mol\,l^{-1}$ dopamine was $385\pm106\,nl\,min^{-1}$ (N=34) and this was set as 100 %. Both neurotransmitters evoked dose-dependent secretion rates within the tested concentration range from 10^{-9} to $10^{-4}\,mol\,l^{-1}$. The maximal secretory response of the glands, stimulated by $10^{-4}\,mol\,l^{-1}$ dopamine, was 108.7±6 % (N=3) of the secretory rate evoked by the standard concentration ($10^{-6}\,mol\,l^{-1}$ dopamine). This value corresponds to a secretory rate of $418\pm23\,nl\,min^{-1}$ (calculated from the average secretion rate of the standard). Secretion in response to serotonin stimulation ($10^{-4}\,mol\,l^{-1}$) reached a maximum of 98.8±11 % of the secretory rate evoked by the standard (corresponding to $379\pm43\,nl\,min^{-1}$, N=4). Thus, the maximal secretory response of the salivary glands to stimulation by serotonin is only 91 % of that produced by dopamine stimulation. Half-maximal secretory responses were achieved at concentrations of $6\times10^{-7}\,mol\,l^{-1}$ serotonin and $1.1\times10^{-7}\,mol\,l^{-1}$ dopamine. These data indicate that

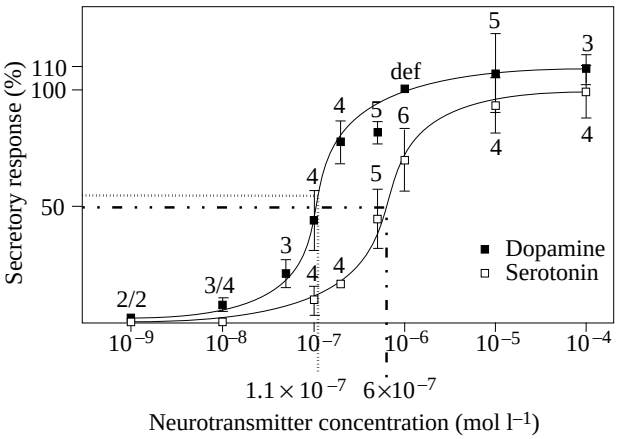

Fig. 3. Dose–response curves for the stimulation of saliva secretion by dopamine and serotonin. The arithmetical mean of the normalized secretory rates evoked by the different neurotransmitter concentrations is given together with the standard deviation and the number of experiments. The secretory response to the standard ($10^{-6}\,mol\,l^{-1}$ dopamine, def) was set at 100 %.

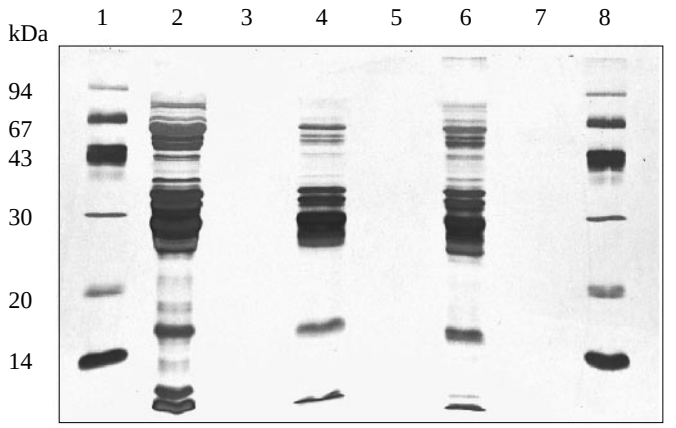

Fig. 4. SDS–PAGE of saliva samples from two individual preparations, stimulated by either 10^{-6} mol l^{-1} serotonin (lanes 2, 4, 6) or 10^{-6} mol l^{-1} dopamine (lanes 3, 5, 7). Lanes 1 and 8 show 10 μl samples of low-molecular-mass marker (Pharmacia) containing phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). Lane 2, 2 μl of serotonin-stimulated saliva (1:2); lane 4, 15 μl (1:100); lane 6, 7.5 μl (1:50); lane 3, 2 μl of dopamine-stimulated saliva (1:2); lane 5, 15 μl (1:100); lane 7, 7.5 μl (1:50).

dopamine is the more potent stimulator of the secretory response of the isolated salivary glands.

Comparison of the protein contents of saliva

Eight individual preparations were stimulated for 20 min by either 10^{-6} mol l^{-1} serotonin or 10^{-6} mol l^{-1} dopamine. The saliva produced was sampled and prepared for protein detection by SDS–PAGE and silver-staining techniques. This protocol is highly sensitive and has been shown to detect proteins in the nanogram range (Blum *et al.* 1987). Fig. 4 illustrates the results from two individual preparations. Microlitre volumes of saliva, produced after serotonin stimulation, showed more than 20 polypeptide bands within a wide range of molecular masses (Fig. 4, lanes 2, 4, 6). In contrast, saliva secreted after dopamine stimulation had no visible protein content (Fig. 4, lanes 3, 5, 7). These results were obtained in all preparations (N=4). Saliva secreted after serotonin stimulation of glands from different cockroaches showed no differences in protein composition, although minor quantitative differences could be observed (data not shown).

Ultrastructural changes in stimulated glands

The anatomy and ultrastructure of the salivary glands in cockroaches are described in detail elsewhere (Kessel and Beams, 1963; Bland and House, 1971; Just and Walz, 1994*a*). In brief, the salivary glands of *Periplaneta americana* consist of numerous secretory acini or 'endpieces' and their ducts. It has been proposed that the ducts modify the primary saliva by reabsorption of ions and water (Just and Walz, 1994*b*,*c*). The acini consist of three morphologically distinct cell types. Structural characteristics indicate that these cells are specialized for the transport of electrolytes and water (peripheral cells), for the production of proteins and mucins (central cells) or have a stabilizing function (inner acinar duct cells). In the unstimulated salivary glands, the peripheral cells always lie in pairs at the base of each acinus. They are characterized by a prominent basal labyrinth and numerous long microvilli facing the lumen of the duct. Mitochondria are abundant in the cytoplasm, particularly between the basal infoldings. The central cells, which follow downstream, contain abundant rough endoplasmic reticulum, dictyosomes and secretory vesicles.

Salivary glands stimulated for either 5 or 20 min by 10^{-6} mol l^{-1} dopamine showed no significant changes in the ultrastructure of the secretory acini and the distal ducts (Fig. 5). In some preparations, the inner acinar ducts seemed to be distended in comparison with those in unstimulated glands. These minor changes, however, were not representative and were difficult to establish quantitatively.

After 5 min of stimulation by 10^{-6} mol l^{-1} serotonin, the central cells showed some exocytotic activity, although secretory vesicles were still abundant throughout their cytoplasm (data not shown). In contrast, dramatic ultrastructural changes could be detected in salivary glands stimulated by 10^{-6} mol l^{-1} serotonin for 20 min (Fig. 6). Most

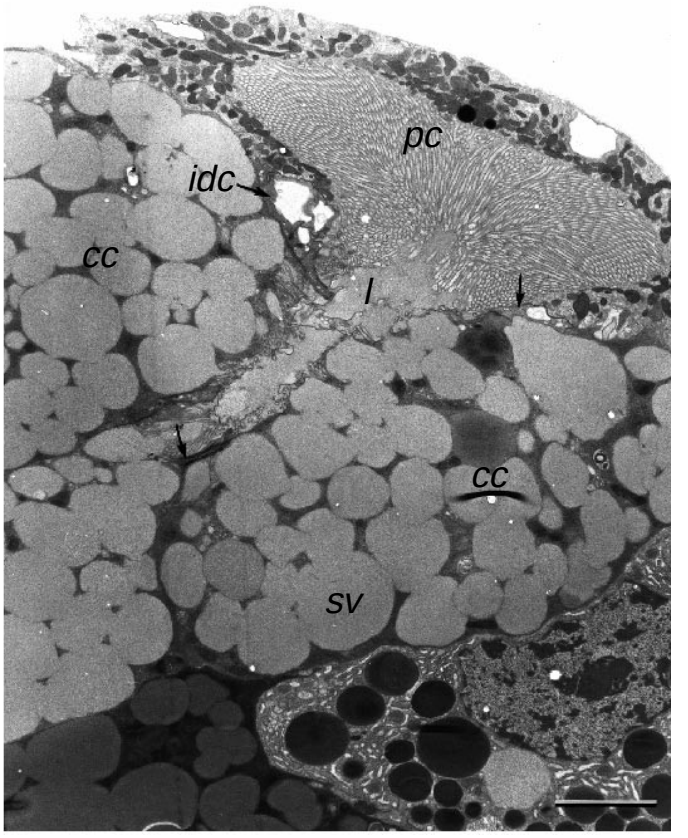

Fig. 5. Longitudinal section through one secretory acinus stimulated for 20 min by 10^{-6} mol l^{-1} dopamine. *cc*, central cell; *idc*, processes of the inner acinar duct cells (arrows); *l*, lumen of the duct; *pc*, peripheral cell; *sv*, secretory vesicle. Scale bar, 4 μm.

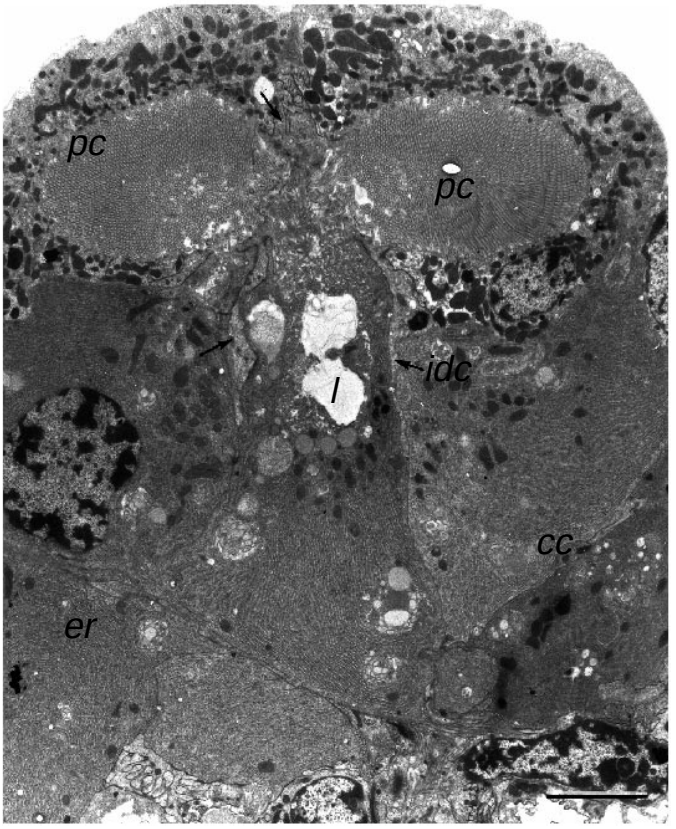

Fig. 6. Oblique section through one secretory acinus stimulated for 20 min by 10^{-6} mol l^{-1} serotonin. *cc*, central cell; *idc*, processes of the inner acinar duct cells (arrows); *l*, lumen of the duct; *pc*, peripheral cell; *er*, endoplasmic reticulum. Scale bar, 4 μm.

central cells had lost their secretory vesicles completely. The other cell types in the glands, that is peripheral cells, inner acinar duct cells and distal duct cells, showed no obvious ultrastructural changes. These data indicate that serotonin stimulates the exocytosis of the secretory vesicles from the central cells, whereas dopamine stimulates only fluid secretion by the salivary glands.

Discussion

The maximal secretory rates measured in isolated salivary glands of *P. americana* are approximately 5–10 times higher than those reported for salivary glands in another laboratory cockroach, *Nauphoeta cinerea* (Table 1). Although *N. cinerea* is about 25 % smaller than *P. americana* (Cornwell, 1968), we do not think that the differences in the rates of saliva secretion are attributable to the size difference. The perfusion media used in the studies with the *N. cinerea* salivary glands were not oxygenated (House and Smith, 1978; Maxwell, 1981; Evans and Green, 1991); we have noted that the maximal secretory rates of isolated salivary glands of *P. americana* drop to 60–100 nl min^{-1} in non-oxygenated physiological saline. Therefore, it seems likely that the differences in secretion rates result from differences in experimental conditions rather than from species differences.

The dose–response curves reported in this study support the thesis that dopamine is the more potent stimulator of the secretory response of the isolated salivary glands (House and Smith, 1978). However, there is a dramatic difference in protein content between saliva samples from dopamine- and serotonin-stimulated glands (Fig. 4). These data show, for the first time, that the two neurotransmitters stimulate the formation of two different types of saliva, so that secretory rates evoked by dopamine and serotonin are not directly comparable.

The ultrastructural changes in the salivary glands in response to stimulation support the hypothesis that only serotonin stimulates the exocytosis of proteins from the central cells. However, we cannot exclude the possibility that serotonin also stimulates the transport of ions and water by the peripheral cells and/or the distal duct cells. Nevertheless, the total amount of fluid secreted upon stimulation by a given dopamine or serotonin concentration is smaller after serotonin than after dopamine stimulation. The observation that the secretory response of the salivary glands to high serotonin concentrations declines within a few minutes (Fig. 2B) may be attributable to a slow depletion of the ‘salivary protein stores’ in the central cells (see also Fig. 6) or may represent an adaptation phenomenon. However, these effects can be observed only at

Table 1. *Secretory rates of isolated cockroach salivary glands*

Species	Stimulation method	Maximal secretory rate (nl min^{-1})	Agonist concentration for half-maximal response (mol l^{-1})	Reference
Nauphoeta cinerea	Electrical	68.6±3.8	–	House and Smith (1978)
	Dopamine	80.6±7.8	0.88×10^{-7}	
	Adrenaline	75.2±8.2	4.4×10^{-7}	
	Noradrenaline	72.3±2.5	7.2×10^{-7}	
	Serotonin	34.5±4.2	2.7×10^{-7}	
Nauphoeta cinerea	Electrical	80.0	–	Maxwell (1981)
Nauphoeta cinerea	Dopamine	126.0	–	Evans and Green (1991)
Periplaneta americana	Dopamine	418±23	1.1×10^{-7}	This study
	Serotonin	379±43	6.0×10^{-7}	

serotonin concentrations above 10^{-6} mol l^{-1}. These are probably unphysiologically high concentrations. In addition, our Ringer's solutions contain no substrates for protein biosynthesis by the salivary gland cells and, therefore, do not provide the same conditions as in the haemolymph of the living animal. Thus, the observed declining secretion rates of the isolated salivary glands may not directly reflect the response of the salivary glands to high serotonin concentrations *in vivo*.

The innervation of the salivary glands of cockroaches and locusts and the identity of the putative neurotransmitters stimulating saliva secretion *in vivo* has been extensively studied (Whitehead, 1971; Bowser-Riley, 1978; Ali *et al.* 1993; Ali and Orchard, 1994; Elia *et al.* 1994). The glands are primarily innervated from the suboesophageal ganglion *via* the paired nerve 7b. Cockroach salivary glands are also innervated from the stomatogastric nervous system (Whitehead, 1971; Gifford *et al.* 1991). Nerve 7b contains two axons, termed SN1 and SN2. These project to the salivary gland complex and branch extensively over the secretory acini (Ali *et al.* 1993). The immunocytochemical localization of tyrosine hydroxylase (the rate-limiting enzyme for the synthesis of dopamine) in SN1 of locusts and cockroaches provides evidence that dopamine is one possible neurotransmitter for the salivation process in these insects (Ali *et al.* 1993; Elia *et al.* 1994). Furthermore, SN2 of locusts has been shown to contain serotonin (Gifford *et al.* 1991; Ali *et al.* 1993). Both neurotransmitters can also be detected by high-pressure liquid chromatography (HPLC) in the salivary glands of locusts, where they have been shown to increase cyclic AMP levels (Ali *et al.* 1993). Although pharmacological data indicate that dopamine and serotonin stimulate the glands *via* different receptors (Ali and Orchard, 1994), the exact localization of these receptors within the salivary gland tissue is unknown.

In general, the salivary glands and their innervation in cockroaches and locusts show a similar morphology, but there are minor differences in the occurrence of the neurotransmitters that are believed to be involved in the formation of saliva. Although it is now generally accepted that dopamine stimulates the salivary glands of cockroaches *via* SN1, the neurotransmitter content of SN2 remains questionable (Gifford *et al.* 1991; Elia *et al.* 1994). Nevertheless, serotonin-immunoreactivity has been localized in the oesophageal nerve, which is the second source of innervation for the salivary glands, and the 'small-diameter axons' accompanying SN2 in the cockroach (Davis, 1985). In addition, the presence of serotonin in the salivary gland complex of the cockroach has been demonstrated by HPLC (Elia *et al.* 1994). We conclude that both dopamine and serotonin are putative neurotransmitters for saliva formation in *Periplaneta americana*.

Taken together, our data reveal that the isolated salivary glands of the cockroach are able to produce different types of saliva. The multiple innervation of the glands and the occurrence of different neurotransmitters in the gland complex indicate that these saliva types can also be produced by the living animal. Thus, the process of saliva formation in insects seems to be more complex than described to date. Like vertebrates, some insects seem to be able to select between protein-rich and watery salivas for different purposes, e.g. in their feeding and cleaning behaviour. The use of different salivary protein concentrations for different foods, for example, would optimize the feeding procedure in the omnivorous cockroaches. In contrast, it would be advantageous for the same insects to clean their antennae with a watery fluid rather than to contaminate them with a sticky saliva, rich in protein and mucin.

The possibility that several different types of saliva can be produced by cockroaches raises the question of how secretion of the different saliva components is coordinated. Knowledge about the neurotransmitter contents of SN2, the small-diameter axons and the axons from the stomatogastric nervous system remains incomplete. Moreover, there is no information available concerning the possible occurrence of neurohormones, e.g. neuropeptides, within the haemolymph of the feeding animal. It therefore seems likely that neurotransmitters and neuromodulators in addition to dopamine and serotonin are involved in the physiology of the salivary glands and that our understanding of saliva formation in the cockroach and in other insects is still in its infancy.

This work was supported by the Deutsche Forschungsgemeinschaft Wa 463/8-1. We should like to thank Professor Dr G. Löffler and M. Schmidt for introducing us to the silver staining techniques and Dr Otto Baumann for critically reading the manuscript.

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