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AN ELECTROPHYSIOLOGICAL STUDY OF NEUROGLANDULAR TRANSMISSION IN THE ISOLATED SALIVARY GLANDS OF THE COCKROACH

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INTRODUCTION

Little is known about the nervous control of salivary secretion in insects. Apparently there have been no electrophysiological studies of the transmission process occurring at neuroglandular junctions. This paper describes electrical responses of cockroach salivary gland cells to nervous stimulation in order to provide a basis for the eventual understanding of neuroglandular transmission in this gland.

METHODS

The cockroach, *Nauphoeta cinerea* Olivier, was used in this study. The insects were reared in a glass aquarium in a heated room and fed on rat cake. Water was provided *ad libitum*.

The structure and function of the salivary glands of *N. cinerea* have been described by Bland & House (1971). Glands were dissected from adult cockroaches and washed in Ringer solution containing 160 mM-NaCl, 10 mM-KCl, 5 mM-$CaCl_2$, 1 mM-$NaHCO_3$ and 0·1 mM-NaH_2PO_4 per litre. The reservoirs and main salivary ducts were removed from the glands which were then sandwiched between two platinum meshes in the experimental chamber (Fig. 1). The apparatus was constructed so that the glands could be perfused with Ringer solution whose composition could be altered without displacing the recording electrode from the cells. Two Ag/AgCl electrodes were located in the base of the chamber; one of these served as an 'earth electrode' for the recording circuit while the other was used as a stimulating electrode.

When the gland was in position between the platinum meshes a glass micro-capillary electrode was inserted into an acinus in order to record the membrane potential of a gland cell. Membrane potentials were recorded differentially between two glass microelectrodes filled with 3 M-KCl; the resistances of the microelectrodes used in this investigation lay in the range 10–20 MΩ. Each microelectrode was connected to the input of a Bak wide-band electrometer with unity gain, and the outputs of the electrometers were fed into a differential amplifier of a Tektronix 502A dual-beam oscilloscope in parallel with a Weir digital voltmeter (Type 500 Mark II).

Nerve stimulation was achieved by passing current between an upper Ag/AgCl electrode placed over the site of recording and the stimulating electrode in the chamber's base. Stimuli were delivered either from a Tektronix pulse generator Type 161

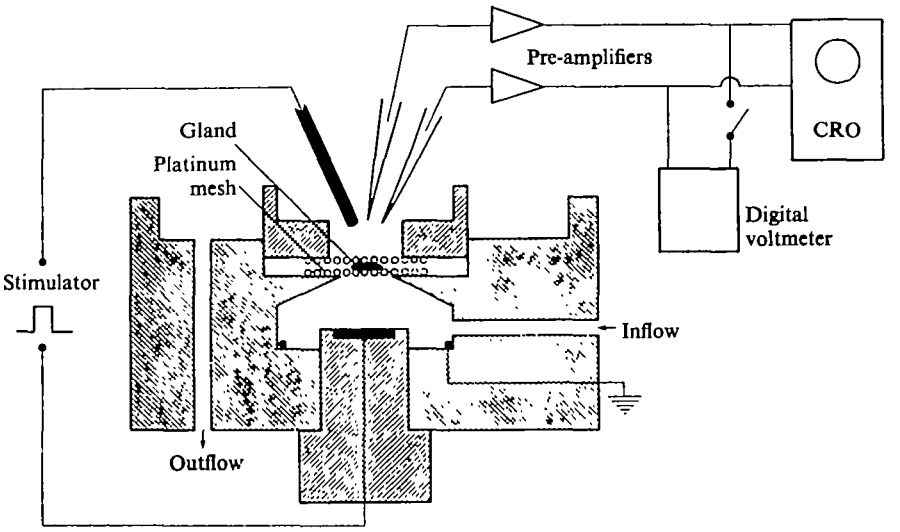

Fig. 1. Schematic diagram of the experimental chamber used for membrane potential measurements in isolated salivary glands.

with an A.E.L. stimulus isolator (Model 112) or a Grass stimulator (Type SD5). The duration of each stimulus lay in the range 1–2 msec and its amplitude in the range 1–90 V.

Photographic records were obtained with a Polaroid camera or a Cossor Instruments Oscillograph camera (Model 1458).

RESULTS

Membrane potential

Upon inserting a microelectrode into an acinus, membrane potentials of about −30 mV were recorded when the gland was bathed in Ringer solution. The membrane potential is conventionally expressed as the potential of the inside with respect to the outside of the cell. Generally the recorded potentials remained approximately constant for long periods of 10–30 min and occasionally for over 60 min; indeed, such stable recordings were necessary for many of the experiments to be described later. Unfortunately the actual recording sites in the individual acini have not been established, but it seems most likely that the potential difference was recorded across the basal membrane of the acinar cells since the potential recorded by the advancing microelectrode frequently dropped suddenly to a new level when it entered the acinus. In the majority of such impalements the potential increased slowly in amplitude over the subsequent 10–30 sec. The acinar cells in this gland are of two kinds – peripheral cells and central cells – and all of the cells, irrespective of type, are joined together by septate desmosomes (Bland & House, 1971). Such intercellular junctions are known to be sites of electrical coupling in *Drosophila* salivary gland (Loewenstein & Kanno, 1964) and, therefore, it is possible that all of the cells in a given acinus of the cockroach salivary gland have the same membrane potential.

An alternative view, that the membrane potentials are recorded between the acinar

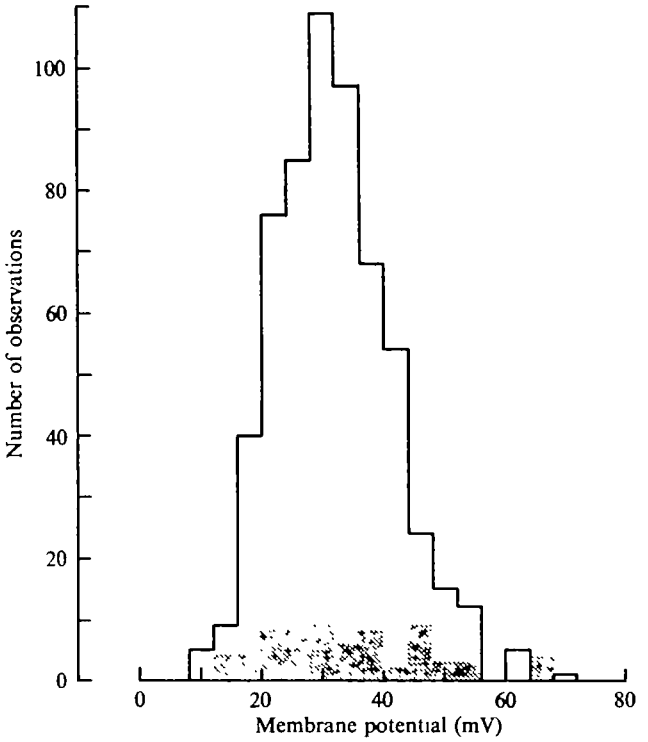

Fig. 2. Distributions of membrane potentials for gland cells. The shaded histogram denotes the values for cells which did not respond to nervous stimulation.

lumen and the bathing solution, seems unlikely because the luminal volume occupies a very small fraction of the total acinar volume in this gland (Bland & House, 1971).

Fig. 2 shows two superimposed histograms of membrane potentials recorded in a series of 668 impalements of different gland cells. Of the total number of cells studied, 68 did not respond to nervous stimulation and their distribution appears as the shaded histogram. There is no difference between the distribution of the membrane potentials recorded in the cells which did not respond, and the distribution in the 600 cells which did. The mean membrane potential ($\pm$ S.E.) for the former is $-34{\cdot}6 \pm 2{\cdot}1$ mV and for the latter is $-32{\cdot}3 \pm 0{\cdot}8$ mV. The difference between the groups cannot be attributed to any factor at present and perhaps it represents failure due to cellular damage under the present experimental regime.

Response to nervous stimulation

In these experiments the salivary nerves were excited by 'field stimulation' which was achieved by passing a current pulse of about 1–2 msec duration across the tissue in the vicinity of the recording electrode. For this type of nerve stimulation the amplitude of stimulating voltage pulse usually exceeded 10 V. The response to a single shock consisted of a transient hyperpolarization which occurred after a latency of about 1 sec. Identical electrical responses have been recorded in the acinar cells of this gland after electrical stimulation of the salivary duct nerves with a suction electrode (C. R. House & B. L. Ginsborg, unpublished), thus verifying that the 'field

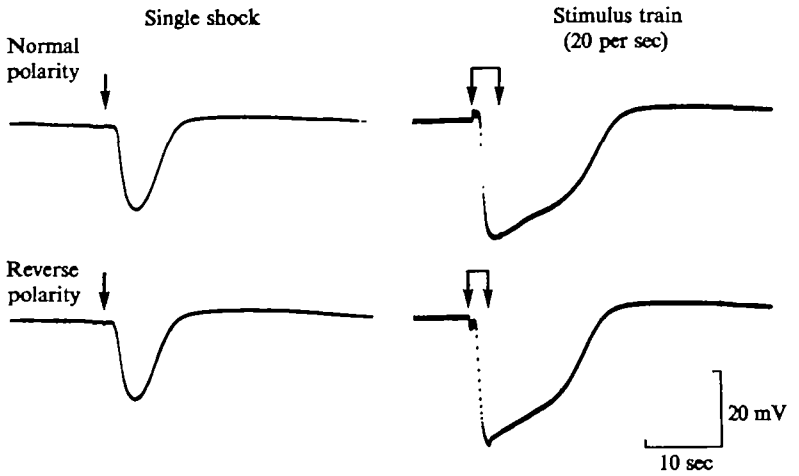

Fig. 3. Typical electrical records from a gland cell showing responses to single shocks and trains of stimuli of either normal or reverse polarity. The gland was bathed in low-potassium Ringer solution ($[K_o]$ = 1 mM) to obtain large responses (cf. Fig. 9).

stimulation' employed in the present experiments excites the salivary nerves. The innervation of the cockroach salivary gland has been described recently by Whitehead (1971).

Both the size and the time course of the electrical responses to 'field stimulation' were independent of the polarity of the stimulating pulses (Fig. 3). The main features of the hyperpolarizing responses of the acinar cells to a single shock are (*a*) an initial latency of about 1 sec, (*b*) a time-to-peak of about 2 sec, (*c*) an overall duration of about 10 sec, and (*d*) an amplitude in the range 1–30 mV. When a train of stimuli was delivered at a rate of 20 per sec the latency remained the same but the amplitude and the duration of the response became larger than the corresponding response to a single shock (Fig. 3).

An examination of the time courses of the decay of several responses to single shocks (Fig. 4) revealed that they were exponentially related to time. The time constants for decay lay in the range 1·5–3·7 sec, which are, of course, considerably larger than any known membrane time constant. The origin of this slow decay is not known, but it certainly cannot be due to the discharge of the cell membrane's capacity.

Often the membrane potential attained a lower (depolarized) value after the hyperpolarizing phase of the response was over. One might draw a crude analogy with electrically excitable membranes and call this late portion of the response an 'after potential'. Subsequently the 'after potential' decayed slowly over a period of about 1–2 min. Such 'after potentials' are evident in the records of Fig. 3, although they were not observed invariably. When they were observed they were generally smaller than 5 mV in amplitude.

In order to study the different features of the response to nervous stimulation it was essential to know the effect of varying the time interval between successive responses on their magnitudes. Consequently a series of double-pulse experiments was performed. Each experiment consisted of determining first the response to a single shock and then after a given interval recording the response to a second shock. Fig. 5

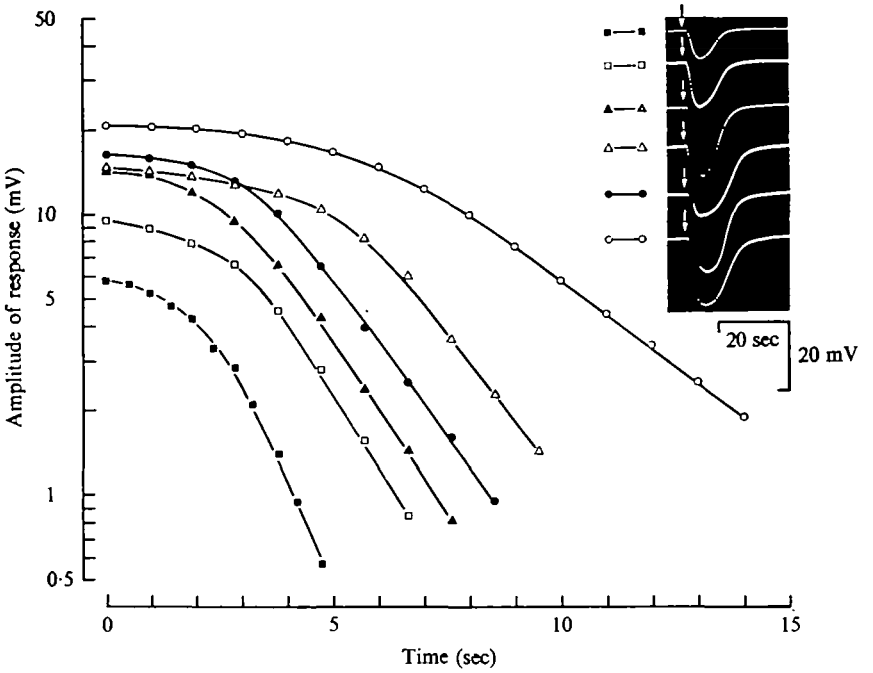

Fig. 4. A semi-logarithmic plot against time of the decay of typical responses to single shocks. The graphs have been obtained from the corresponding records shown in the inset display. The correspondence between each plot and its original trace is denoted by a specific symbol.

shows the results of these experiments, and the upper part of the figure displays typical records from one cell during the double-pulse experiment; the arrows indicate the delivery times of the stimuli. The double-pulse experiments demonstrated that if the interval between identical stimuli were less than 120 sec the second shock elicited a smaller response than the first. This effect has been expressed quantitatively in terms of the ratio of the test response (R_2) to the conditioned response (R_1) and Fig. 5 shows how (R_2/R_1) depends on the interval. Each point is the mean value for 20 cells and the bars indicate $\pm$ S.E. At intervals below 10 sec the ratio (R_2/R_1) was difficult to assess because the responses merge into one another. The analytical procedure used in that case was to record the response to a single shock then wait for 120 sec before recording the double-pulse response; the size of R_2 was obtained by subtracting the original single response from the record of the double-pulse response. Although this is not an entirely satisfactory procedure it is probably accurate enough to indicate that (R_2/R_1) at 5 sec is significantly larger than at 10 sec intervals. No conclusion can be drawn as to whether the depression of the test response originates either presynaptically or postsynaptically. Certainly the phenomenon is not due simply to a progressive decline in the amount of transmitter released by successive stimuli, otherwise the response to a train of stimuli would not be larger and longer than that to a single shock. Moreover, if one delivers three shocks at certain intervals, say 10 sec, then the third response is not invariably smaller than the second response although the second is always smaller than the first.

Given the experimental condition that the interval between successive stimuli

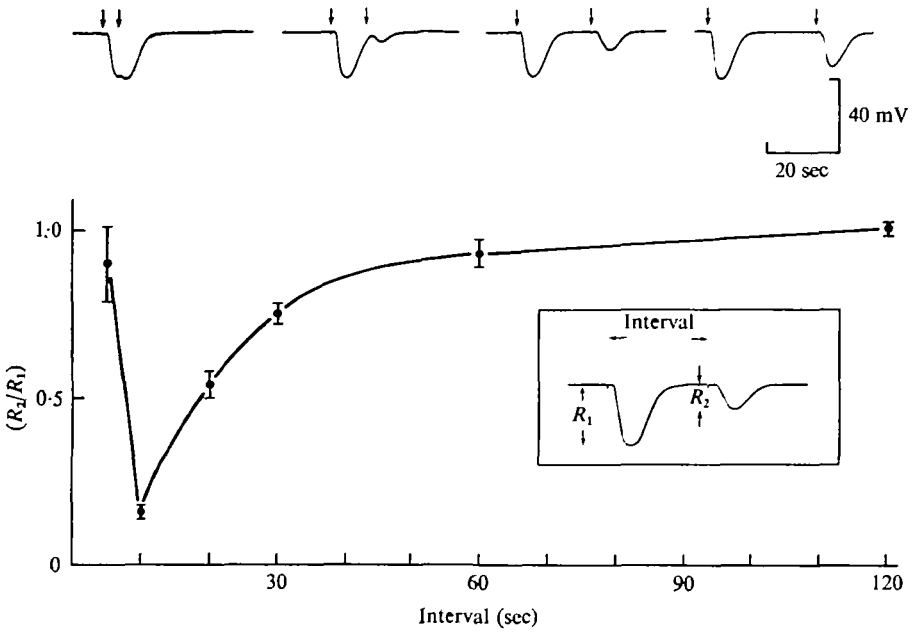

Fig. 5. The results of the double-pulse experiments showing the interaction between stimuli. The amplitudes of the conditioned (R_1) and test (R_2) responses were obtained as shown in the insert diagram. The gland was bathed in low-potassium Ringer solution ([K_0] = 1 mM) to obtain large responses (cf. Fig. 9).

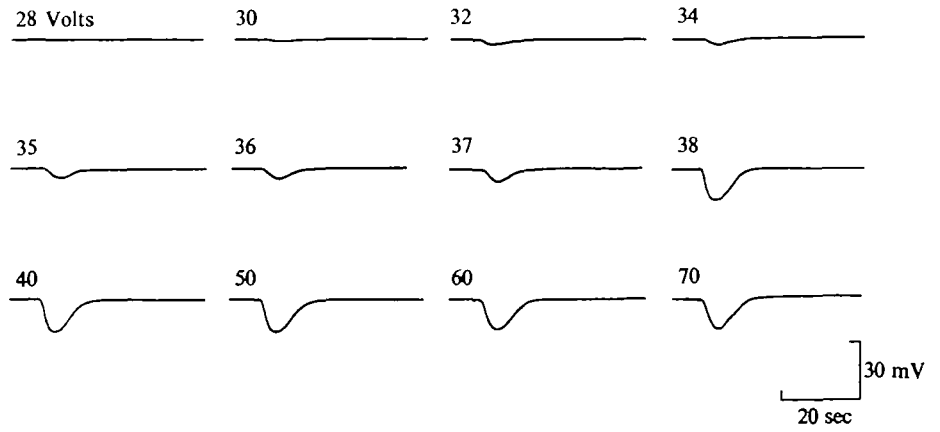

Fig. 6. The relation between stimulus strength and the size of responses to single shocks. The gland was bathed in low-potassium Ringer solution ([K_0] = 1 mM) to obtain large responses (cf. Fig. 9).

ought to be larger than 120 sec it was possible to examine the effect of stimulus strength on the size of the response without any apparent interaction between single shocks confusing the interpretation. Fig. 6 shows how the stimulus strength influenced the amplitude of the response; throughout this experiment the duration of the stimulating pulse was 2 msec. When the stimulus amplitude was below 30 V in this experiment no response was detected, but stimuli above that threshold evoked re-

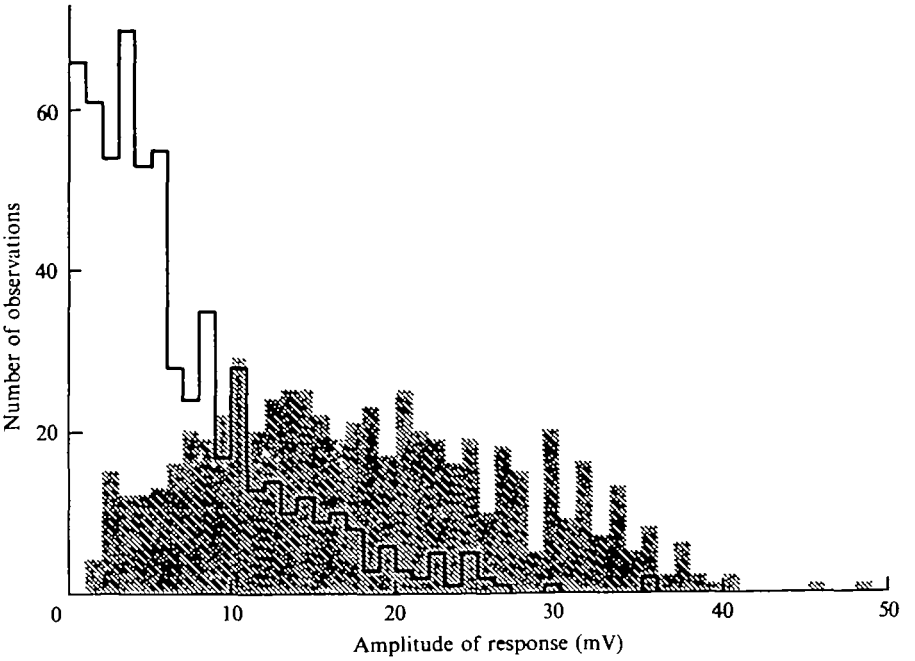

Fig. 7. The distributions of the responses to single shocks and stimulus trains. Stimuli were applied at a rate of 20 per sec.

sponses which showed some grading with stimulus amplitude. The grading of the response was not a smooth function of stimulus intensity and these records indicate that the given gland cell was influenced by transmitter release from several nerves which do not all make the same contribution to the total response. However, it cannot be inferred from such data that a single cell is innervated by several axons because of the possible role of electrical coupling between the cell and its neighbours. The main purpose of this experiment was to establish the stimulating conditions for maximum responses. The experiments reported in the rest of this paper were performed with supramaximal stimuli of 50–90 V with durations of 1–2 msec.

Such supramaximal stimuli were delivered also to the 600 cells referred to previously in Fig. 2. The mean ($\pm$S.E.) amplitudes of the responses recorded in those cells were $6{\cdot}2 \pm 0{\cdot}5$ mV (single shock) and $17{\cdot}5 \pm 0{\cdot}7$ mV (stimulus train) when the glands were bathed in Ringer solution. The distributions of the amplitudes are shown in Fig. 7 where the shaded histogram refers to stimulus trains and the unshaded histogram to the responses to single shocks. No correlation was found between the amplitude of the response and the initial membrane potential.

Effect of stimulating rate on response

Whitehead (1969) has reported that the efferent discharge in the salivary duct nerves controls the rate of salivary secretion in the cockroach *Periplaneta americana*. He recorded the salivary secretion resulting from different rates of efferent discharge and he noted that optimal secretion was associated with a rate of about 6 impulses per second. Fig. 8 shows the electrical responses of a typical gland cell to different rates

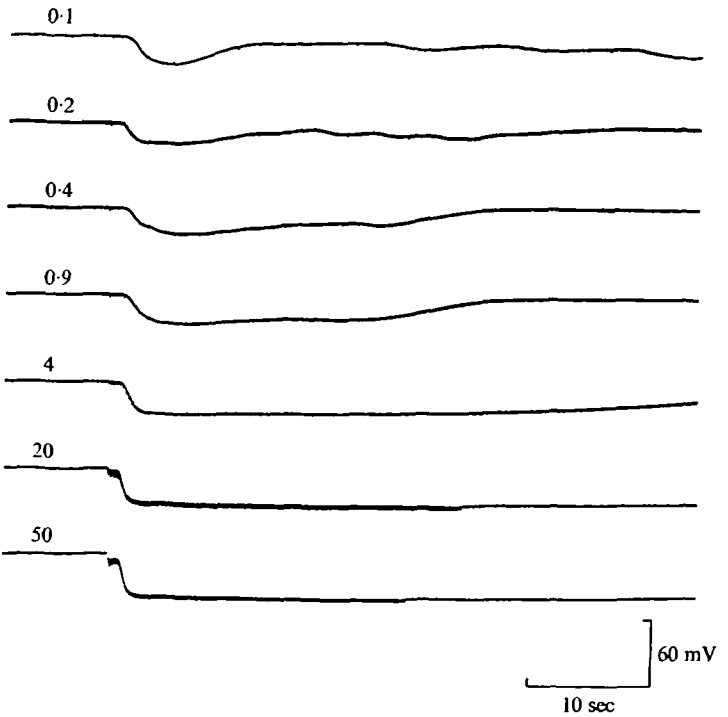

Fig. 8. Effect of the rate of stimulation on the response of a typical cell. The numbers on each record signify the number of stimuli per second.

of stimulation. The numbers on each record denote the number of stimuli per second and the recordings were made with 300 sec rest intervals between each successive stimulus train. At relatively low rates (< 1 per sec) the membrane potential did not attain a new steady value during the stimulus train but rather it tended to oscillate about some value lower than the initial response. However, when the stimulating rate (4–50 per sec) was equal to or above that reported by Whitehead (1969) the cell became hyperpolarized towards a new almost stable level which is presumably associated with salivary secretion. The relationship between the electrical response and salivary secretion remains to be explored. From the data in Fig. 8 and that of similar experiments it was concluded that a suitable rate of stimulation was 20 stimuli per sec and this rate has been used routinely.

Dependence of response on external potassium

Although the cellular concentrations of the principal cations and anions are not known it seems likely that the peak value of the response to nervous stimulation may be close to the equilibrium potential for potassium ions, and a study was therefore made of the dependence of the response on the external potassium concentration $[K_0]$. The upper part of Fig. 9 shows typical responses recorded in glands bathed in Ringer solutions containing different values of $[K_0]$ ranging from 1 to 20 mM. Clearly the amplitude of the response depends on $[K_0]$. The lower part of Fig. 9 shows the results of a series of experiments on 400 gland cells. In these experiments glands were bathed in solutions containing a given value of $[K_0]$ and the membrane potentials at

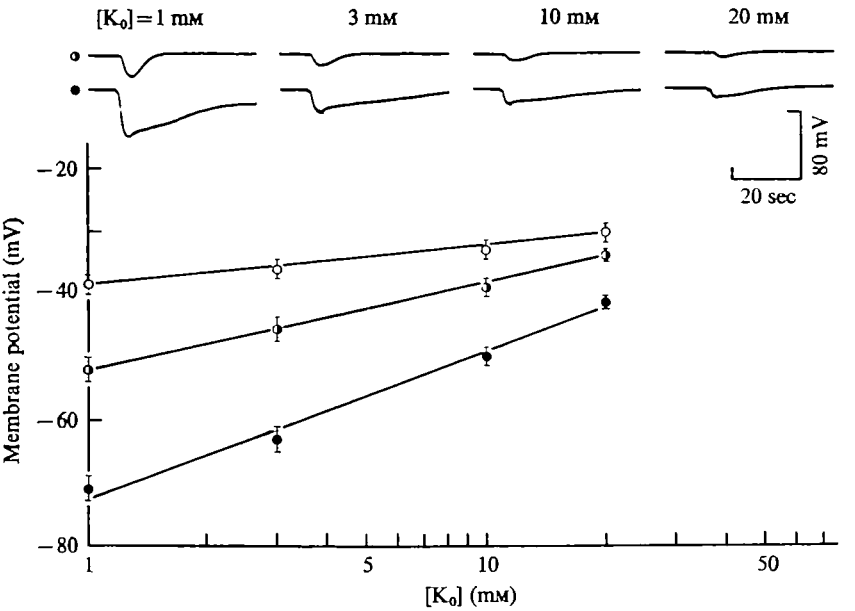

Fig. 9. Effect of external potassium concentration on the membrane potential before and after stimulation. Initial values of the membrane potential are denoted by ○ while the peak values of the responses to single shocks and stimulus trains are denoted by ◑ and ● respectively. The upper part of the figure shows some typical recordings made during this experiment. Stimuli were delivered at a rate of 20 per second.

rest and at the peaks of the responses to single shocks and stimulus trains were recorded for several cells. Each point in Fig. 9 is the mean of 100 observations and the bars indicate ± S.E. values. The dependence of the initial membrane potential on $[K_0]$ is relatively weak, whereas during the responses to nervous stimulation the membrane's permeability to potassium is evidently increased. The increased dependence of the membrane potential on $[K_0]$ was more pronounced in the responses to stimulus trains than in those to single shocks. It seems likely that the electrical response of cockroach salivary gland cells is generated at least partially by an increase in the potassium permeability of the gland cells, although the alternative possibility, that the amount of transmitter released depends on $[K_0]$, cannot be ruled out.

Electrical response to 5-hydroxytryptamine

Berridge & Patel (1968) have reported that the salivary gland of the blowfly is stimulated to secrete by extremely low concentrations (10^{-9} M) of 5-hydroxytryptamine (5-HT). In the blowfly the salivary gland is not innervated and Berridge and Patel speculated that 5-HT or some similar substance might act as a hormone to stimulate secretion. Whitehead (1969) has shown that 10^{-9} M 5-HT stimulates salivary secretion in cockroach salivary gland and it was therefore considered important to record the effects of 5-HT on the membrane potential of the gland cells. Figure 10 shows the typical effect of 5-HT on the membrane potential. In this experiment the response to a stimulus train was recorded first (trace *a*). Then the gland was perfused at a slow rate (approx. 2 ml per minute) with Ringer solution containing $2{\cdot}5 \times 10^{-7}$ M 5-HT which

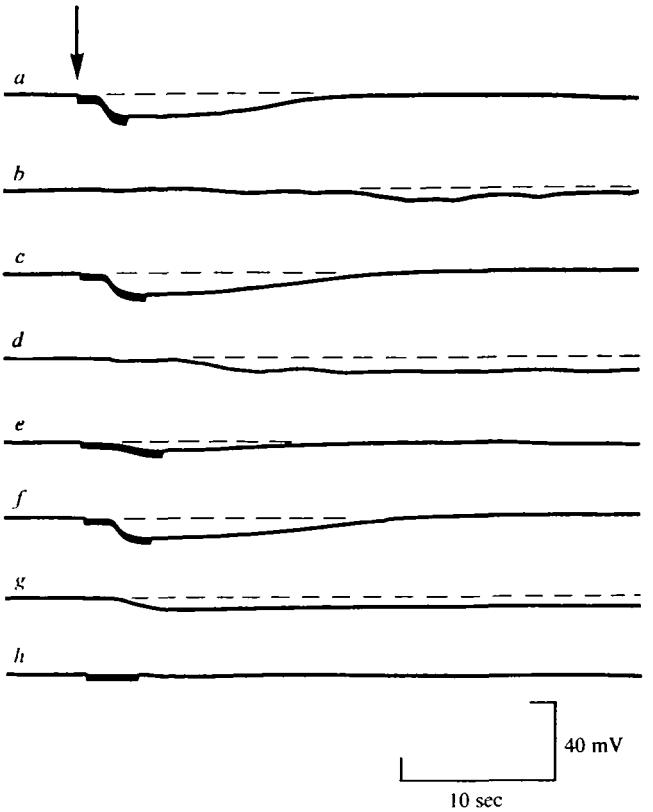

Fig. 10. Electrical responses of a gland cell to stimulus trains and to 5-hydroxytryptamine. Stimuli were delivered at a rate of 20 per second. See text for description of the experiment.

generated a hyperpolarization (trace *b*). After an interval of about 120 sec the response to a stimulus train was recorded (trace *c*) and subsequently the preparation was perfused with 25×10^{-7} M 5-HT (trace *d*). The response to a stimulus train after a further 120 sec was relatively small (trace *e*). The preparation was then perfused with Ringer solution. The response to a stimulus train recovered its normal amplitude (trace *f*). Finally the application of 5-HT at a concentration of 250×10^{-7} M generated a hyperpolarization (trace *g*) but during the presence of 5-HT at that concentration a response to nervous stimulation could not be elicited (trace *h*). After perfusing the gland with Ringer solution the response to nervous stimulation was recorded again in this cell, although it is not displayed in the figure. These data strongly suggest that 5-HT and the neurotransmitter act on the same pharmacological receptors. Of course, this does imply that 5-HT is the neurotransmitter.

It is interesting to note that Berridge & Prince (1972) have found that 10^{-8} M 5-HT generates a negative potential between the lumen of the blowfly salivary gland and the external medium. Recently Prince & Berridge (1972) have shown that this transepithelial response is generated partially by a hyperpolarization of the basal membranes.

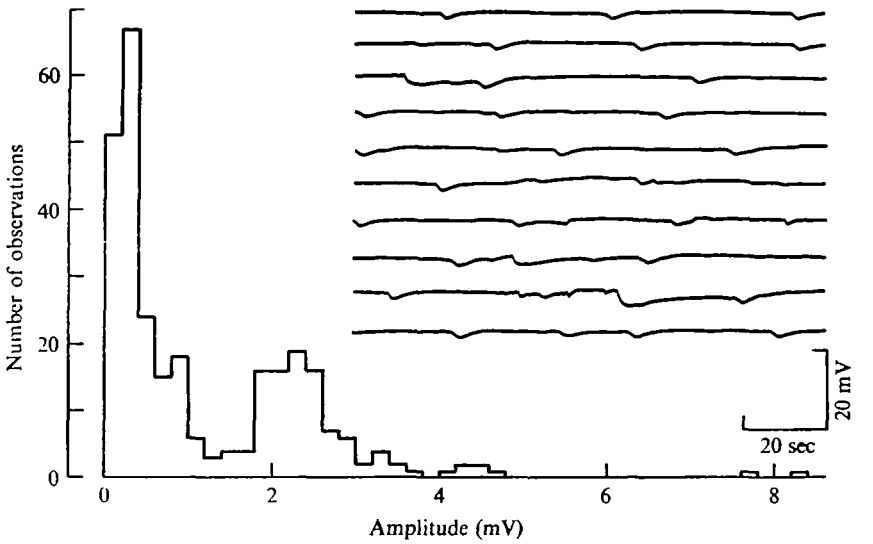

Fig. 11. Distribution of spontaneous changes in the membrane potential of a gland cell. The inset display shows a short continuous excerpt from this experiment.

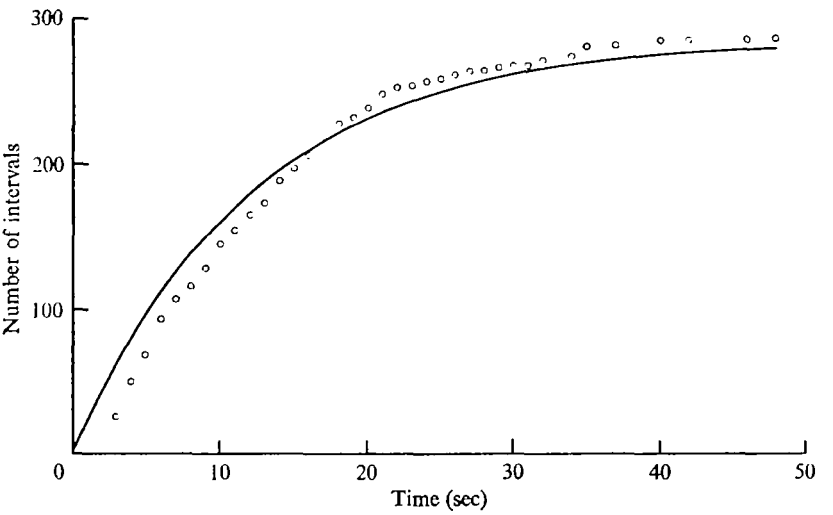

Fig. 12. The number of intervals smaller than a given time t as a function of time. The curve has been obtained from the relation $N[1-\exp(-t/T)]$ cited by Fatt & Katz (1952), where N is the total number of intervals and T is the mean interval between the spontaneous potential changes. In this experiment $N = 288$ and $T = 12{\cdot}3$ sec.

Spontaneous changes in membrane potential

During the course of many experiments the membrane potentials of some cells underwent spontaneous changes of sign and duration similar to those of typical responses to single shocks. The spontaneous activity was sufficiently persistent in only three cells out of over 1000 for its characteristics to be analysed. The reason for this small number of experiments is that the mean interval between the successive spontaneous changes in membrane potential is about 12 sec and consequently the recording

time required for an acceptable number, say 300, of observations in a given cell is 60 min at least. Fig. 11 shows the results of one such experiment and the insert is a short continuous excerpt from the photographic record. The distribution of amplitudes of the spontaneous potential changes indicates that there are apparently two different populations of spontaneous potentials. These changes might be 'miniature potentials' arising from the spontaneous release of transmitter from the nerve terminals. One way of testing that hypothesis is to examine the intervals between the so-called 'miniatures' to see if they occur randomly. This analytical procedure was first employed by Fatt & Katz (1952) for the miniature end-plate potentials at the skeletal neuromuscular junction. Fig. 12 shows the total number of intervals with durations smaller than any time t plotted against time and also the theoretical curve for a random process. It is clear that the experimental data cannot be adequately described by a truly random process. Thus, the spontaneous potentials probably do not arise because of a random release of transmitter from a single nerve terminal. It may be that at least two nerve terminals are spontaneously releasing transmitter at different mean rates on the same cell or on several cells which are electrically coupled to one another.

DISCUSSION

The results of this investigation bear a strong resemblance to those of other studies of neuroglandular transmission in vertebrates. These basic areas of similarity will be discussed first before the divergent pieces of information are presented.

Numerous workers have measured the membrane potentials of salivary gland cells, and Table 1 illustrates some representative values. The potentials recorded in the cockroach salivary gland are exceedingly close to the other reported values. Indeed, the salivary gland cells of both vertebrates and invertebrates seem to be characterized by their relatively low resting potentials. The responses to nervous stimulation, or the so-called 'secretory potentials', are also quite similar in the different species with the exception of some units in the cat's mandibular gland where depolarizing as well as hyperpolarizing responses have been recorded.

An interesting feature of the neuroglandular transmission processes in all of the innervated glands shown in the table is that they involve latencies in the range 0·25–2·0 sec. No one has been able to account for such long delays in the transmission process. It is highly unlikely, for example, that the conduction time in the nerves of the mammalian salivary glands represents a significant fraction of the latency. For instance, Creed & Wilson (1969) have shown that the conduction time along the parasympathetic preganglionic fibres is about 12 msec in the cat submandibular gland and, furthermore, they have argued that the ganglionic delay is probably less than 30 msec. In the cockroach also it seems highly unlikely that the conduction time is long enough to explain the delay especially since the 'field stimulation' employed in my experiments probably excited the nerve terminals directly. Another possible source of delay would arise if the transmitter had to diffuse a relatively long distance, x, between its point of release from the nerve and the gland cell membrane. In this case the time required for diffusion would be given by $x^2 = 2Dt$, where D is the diffusion coefficient of the transmitter. If one assumes that the transmitter is a relatively small molecule and has a diffusion coefficient of about 10^{-5} cm sec^{-1} then the

Table 1. *Some electrical characteristics of salivary gland cells*

Preparation	Membrane potential (mV)	'Secretory potential' Latency (sec)	Amplitude (mV)	Duration (sec)	Comments	Reference
Fruit fly larva	−13	—	—	—	Not innervated	Loewenstein & Kanno (1963)
Blowfly	−44	—	—	—		Prince & Berridge (1972)
Cockroach	−32	1·0	−6	10	Response to single shock	Present study
Cat submandibular						
Type I	−22	0·2	−30	1		Lundberg (1955)
Type II	−32	0·4	−20	10		Lundberg (1955)
Type III	−80	2·0	+60	> 20	Response to stimulus train	Lundberg (1955)
Cat sublinqual	−33	1·0	−30	> 5		Lundberg (1957*a*)
Dog submandibular	−40	0·4	−15	> 5		Yoshimura & Imai (1967)

diffusion path would need to exceed 40 μm to account for a latency of 1 sec. Moreover, according to that view one might expect to see some correlation between the size of the 'secretory potential' and its time-to-peak; this particular point was examined in the present data and no such correlation was found. Thus, a purely diffusional source of delay in transmission does not seem likely to produce the observed latencies.

Very little can be said about the ionic basis of the 'secretory potential' in the cockroach salivary glands except that it may be generated, at least partially, by an increase in potassium permeability. Of course, such a hypothesis needs to be substantiated by other lines of evidence, such as membrane conductance measurements. In the salivary glands of vertebrates, however, there is more information about the role of ionic movements during the 'secretory potential'. For instance, Lundberg (1958) estimated that the equilibrium potentials for the basal membrane of the cat's sublingual gland are: $E_K = -97$ mV, $E_{Na} = +29$ mV and $E_{Cl} = -12$ mV. These values indicate that potassium, sodium, and probably chloride ions also, are not at equilibrium across the basal membrane under resting conditions. The 'secretory potential' might originate from an increase in the potassium permeability which would cause the membrane potential to shift towards E_K. That interpretation is compatible with the efflux of potassium that occurs during the activation of gland cells (Burgen, 1956). Furthermore, according to Lundberg (1957*b*) the specific resistance of the basal membrane drops from 18 to 9 Ω cm^2 during the 'secretory potential'. In apparent conflict with those lines of evidence is the observation (Lundberg, 1957*b*) that the amplitude of the 'secretory potential' was not reduced when the membrane potential was raised to more than −100 mV. Consequently Lundberg (1957*b*) concluded that the 'secretory potential' is generated by an active influx of chloride ions. His hypothesis was compatible with the additional finding that both the rate of salivary secretion and the amplitude of the 'secretory potential' were reduced when external chloride was replaced by nitrate, iodide or thiocyanate (Lundberg, 1957*c*). However, Imai (1965) and Yoshimura & Imai (1967) demonstrated that 'secretory potentials'

in the canine submandibular gland could be abolished by raising the external potassium concentration above 13 mM and yet nevertheless nervous stimulation still elicited salivary secretion. They also found that replacement of external chloride with sulphate anions abolished salivary secretion, whereas 'secretory potentials' could still be evoked. The work of Yoshimura & Imai demonstrated, therefore, that the 'secretory potential' was not obligatorily linked to salivary secretion nor was it due to active chloride transport in particular. Instead these authors suggested that the 'secretory potential' was generated by an increase in the potassium permeability since an increase in $[K_0]$, as has already been mentioned, decreased the response, whereas reducing $[K_0]$ below its normal value increased its amplitude. If this is the case, then the observation (Lundberg, 1957*b*) that the 'secretory potential' is apparently independent of the membrane potential remains to be explained.

Apart from the preceding points of similarity between certain aspects of neuroglandular transmission in the cockroach and some vertebrates there is one notable difference. In the cockroach salivary gland there is some depression (of unknown origin) of the response to a second stimulus delivered within quite long intervals after the first; this does not occur, for instance, in the cat's submandibular gland (Lundberg, 1955).

Probably the main point of divergence in neuroglandular transmission in the insects from that in the vertebrates will be the identity of the neurotransmitter. Unfortunately no conclusions about the transmitter in cockroach salivary gland can be drawn from my experiments but this problem is under study.

SUMMARY

1. Some aspects of neuroglandular transmission in isolated salivary glands of the cockroach have been studied.

2. The membrane potential of acinar cells is $-32{\cdot}3 \pm 0{\cdot}8$ mV (mean $\pm$ S.E; $N = 600$ cells) when the gland is bathed in Ringer solution.

3. Upon delivering a single shock by 'field stimulation' to the salivary nerves the gland cell membrane undergoes after an initial latency of 1 second a transient hyperpolarization of about 1–30 mV which lasts for about 10 sec.

4. When the salivary nerves are stimulated by trains of current pulses the hyperpolarization that occurs is larger in amplitude and longer in duration than that after a single stimulus.

5. The amplitude of the responses to single shocks and stimulus trains depends on the external potassium concentration. Thus, the neurotransmitter may increase membrane permeability to potassium ions.

6. The electrical response of the gland cell to 5-hydroxytryptamine in concentrations from $2{\cdot}5 - 250 \times 10^{-7}$ M is similar in sign and magnitude to that of nervous stimulation.

7. Occasionally small fluctuations in the membrane potential are observed and these are similar in sign and duration to responses elicited by single shocks to the salivary nerves.

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