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STUDIES ON LOCUST RECTUM

I. STIMULANTS OF ELECTROGENIC ION TRANSPORT

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SUMMARY

1. Homogenates of whole corpora cardiaca (CC) cause increases in the short-circuit current (I_{sc}) and transepithelial electropotential difference (PD) across locust recta of 3-fold and 1·7-fold respectively, in comparison with the values for unstimulated steady-state recta. Maximum stimulation restores rectal I_{sc} and PD to levels observed immediately after removing this organ from animals.

2. Cyclic-AMP causes a similar maximum increase in I_{sc} and PD; however, the response exhibits a much shorter lag time and a faster rate of rise than is observed for stimulation with CC.

3. The addition of CC to the haemocoel side of everted rectal sacs caused whole tissue levels of cAMP in this organ to increase 3-fold.

4. The relationship between the logarithm of CC or cAMP concentration and the increase in I_{sc} is linear, and the decline in ΔI_{sc} with time is also dose–dependent.

5. Small maximum increases in I_{sc} are caused by homogenates of ventral ganglia, whole brain and rectal tissue, but the concentration of the stimulatory activity in these locust tissues is clearly three orders of magnitude lower than in CC.

6. Inhibitors of HCO_3^-/H^+ and Cl^- transport in vertebrate systems, acetazolamide and thiocyanate, do not inhibit the stimulation of recta by CC or cAMP.

INTRODUCTION

Haemolymph composition in most terrestrial insects is ultimately regulated by selective resorption in the rectum from a fluid secreted by the Malpighian tubules (reviewed by Maddrell, 1971; Phillips, 1977). Phillips (1964*a*, *b*) showed that rates of ion absorption from recta *in situ* are considerably reduced and the rate of water absorption increased when hydrated locusts are fed concentrated saline solutions; clearly these transport processes are regulated. Recent studies have demonstrated that the steady-state transport of water across this epithelium can be driven by any one of Cl^-, Na^+ or K^+ (Phillips, 1977; Goh & Phillips, 1978). Therefore, diuretic or antidiuretic factors might act by regulating the transport of these ions. Williams *et al.* (1978) noted a rapid initial decline in active Cl^- transport and I_{sc} across voltage-

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clamped recta *in vitro* before a steady state was reached. They speculate that this is a consequence of removing recta from a neural or humoral stimulant which is present *in vivo*.

We are not aware of any previous reports of neural or endocrine factors which directly influence rectal transport of ions. There is some evidence, however, for the presence of diuretic and antidiuretic factors in various neural and endocrine tissues of insects (reviewed by Gee, 1977). These factors alter rates of fluid absorption by *in vitro* recta, but evidence that they are natural regulatory agents of rectal transport *in vivo* is not conclusive. In locusts, the storage lobes of the corpora cardiaca (CC) contain a diuretic factor which inhibits rectal absorption of water (Mordue, 1969, 1970), and an antidiuretic factor is present in the glandular lobes (Mordue, 1970). There is some disagreement as to whether homogenates of whole CC exert a diuretic (Mordue, 1970) or an antidiuretic (Cazal & Girardie, 1968) effect on *Schistocerca* recta *in vitro*. Corpora allata of *Schistocerca* have neither diuretic nor antidiuretic activity (Mordue & Goldsworthy, 1969). Such factors are not restricted to the neurohaemal organs. In particular, there is evidence for the presence of antidiuretic factors in the ventral ganglia of *Schistocerca* (see Gee, 1977). Ultrastructural studies have revealed that neurosecretory axons terminate in the rectal wall of other insects (Johnson, 1963, 1966; Gupta & Berridge, 1966; Oschman & Wall, 1969). It is possible therefore that rectal absorption is modified by local release of neurosecretory products. We have already reported (Spring, Hanrahan & Phillips, 1978) that known or putative neurotransmitter substances do not stimulate electrogenic ion transport by locust rectum.

The voltage-clamped preparation of locust rectum developed by Williams *et al.* (1978) offers a rapid method of assaying factors which might control electrogenic transport processes. In this paper, we report the effect of various tissue homogenates, including those reputed to contain diuretic and antidiuretic activities, on the steady-state short-circuit current (I_{sc}) and open-circuit electropotential difference (PD) across *in vitro* preparations of locust recta. We also report the effects on I_{sc} of homogenized rectal tissue, and some inhibitors of anion transport. A preliminary note summarizing the major conclusions in this and subsequent papers has been published (Spring *et al.* 1978).

MATERIALS AND METHODS

The experimental animals were adult *Schistocerca gregaria*, one to three months past their final moult. They were reared at 28 °C and 50% relative humidity under a photoperiod of L:D 16:8 and fed a diet of lettuce and a mixture of dried grass, bran, yeast and powdered milk. *In vitro* preparations of locust recta were obtained from females because of their larger size. Tissue homogenates to be assayed for their ability to influence rectal transport were prepared from adult males to avoid cyclic changes associated with female reproduction.

Electrical measurements in vitro

To measure electrogenic ion transport by the voltage-clamp method, recta were mounted as flat sheets between two 'Ussing-type' perspex chambers as described by

Williams *et al.* (1978). Each chamber contained 7·0 ml of saline which was vigorously stirred by bubbling with 95% O_2–5% CO_2. All experiments were conducted at room temperature (22 °C). The short-circuit current (I_{sc}) was recorded continuously on a 'Fisher Recordall 5000 series' chart recorder. The open-circuit transepithelial potential difference (PD) was monitored at intervals by stopping the voltage clamp for 30 s and using an alternate circuit to record the voltage difference. The membrane d.c. resistance was calculated from the open-circuit PD and I_{sc} using Ohm's Law.

Solutions

A simple insect saline was used to bathe recta in all experiments: 185 mM-NaCl, 11 mM-KCl, 10 mM glucose, 3 mM L-glutamate, 2·5 mM-$MgSO_4$, 1 mm-KH_2PO_4, 24 mM-$NaHCO_3$ and 3 mM-$CaCl_2$. Phenol red was added as a pH indicator. Initial pH was 7·4. Bubbling with the gas mixture reduced this to 7·0 within 5 min. There was no substantial change in pH over the next 4–18 h.

Homogenates of locust tissues

Corpora cardiaca, ventral ganglia, brains, or recta were removed from male locusts with watch-maker's forceps and homogenized in simple insect saline using a Potter–Elvehjem homogenizer, as described by Spring (1979). Homogenates were prepared at a concentration of 1 whole organ per ml. They were made up each day, stored on ice and assayed within 4 h. As a control tissue, a piece of flight muscle several times the size of a ventral ganglion was treated in the same manner as the ganglia.

Electrical stimulation

The *in vitro* rectum was stimulated electrically by disconnecting the voltage clamp and attaching a 'Grass' stimulator directly to the current electrodes. Electrical pulses of varying voltage, frequency and duration were applied across the preparation. The voltage clamp was subsequently reapplied and the I_{sc} measured.

Assay procedure

Small volumes (20–200 μl) of drugs and homogenates were added to the haemocoel side of recta when they had reached the steady-state phase (i.e. 90–150 min after dissection). After testing their effect, the chamber was rinsed with four changes of fresh saline and the I_{sc} across the preparation was allowed to return to a steady-state value before further testing. Preparations were not used for more than three assays (in random order) because the response to standard doses of stimulants subsequently declined. After the final test on each preparation, 0·05 pr CC was added to the preparation to confirm that the tissue still gave a normal response to stimulation. Results from those rare preparations which failed to respond to CC were discarded.

Locusts used for any one experiment usually came from the same cage; i.e. the population had a very limited parentage. We attribute small differences in average values of electrical parameters between experiments done at different times (e.g. Fig. 1 *v.* Fig. 2, first 90 min) to differences in phenotype and to variation in time from last meal.

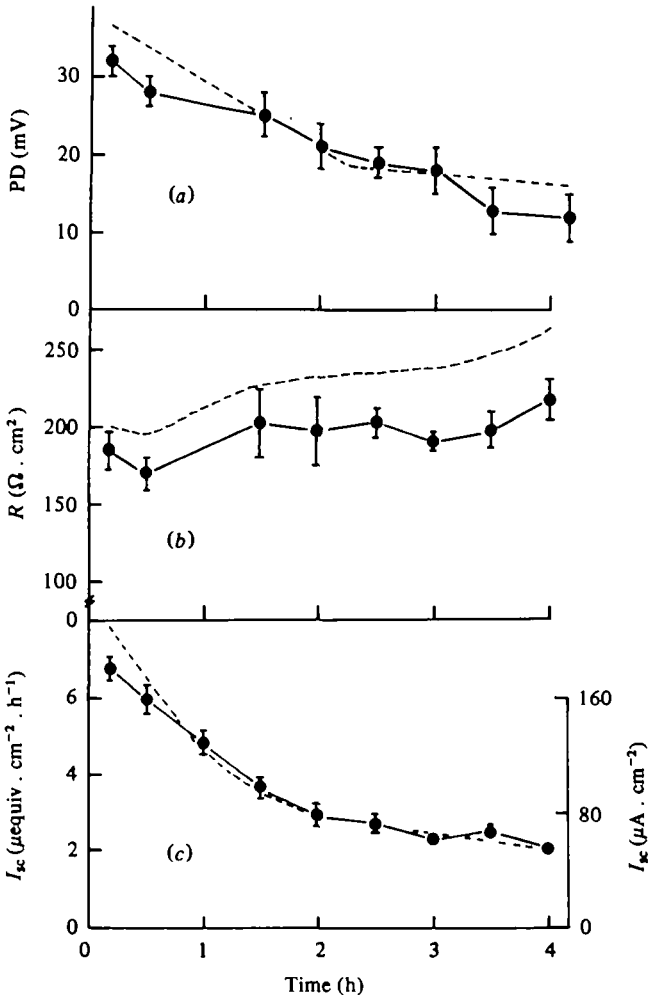

Fig. 1. Viability of unstimulated short-circuited recta in simple Cl-saline as indicated by electrical parameters (mean ± S.E.M., $n = 8$). (*a*) Transepithelial PD (lumen positive). (*b*) Transepithelial d.c. resistance. (*c*) Short-circuit current indicating transport of negative charges L → H (lumen to haemocoel). Dashed lines on all graphs indicate mean values reported by Williams *et al.* (1978) for short-circuited recta bathed in complex Cl-saline.

Tissue cAMP assay

To measure tissue levels of cAMP, everted rectal sacs were prepared from adult female locusts as described by Goh & Phillips (1978). They were weighed and incubated at 37 °C for at least 2 h in simple saline so that recta were in the steady-state phase of fluid transport (Goh & Phillips, 1978). The sacs were then emptied, reweighed, and refilled with 10 μl of simple chloride saline (control) or 10 μl of saline containing 0·25 pr CC and incubated for 15, 30 or 60 min. Controls were incubated for the whole 60 min. The sacs were drained, blotted dry, weighed and then quickly

frozen on pieces of aluminum foil on dry ice. Cyclic-AMP levels were assayed using a competitive binding assay kit (TRK 432) from Amersham/Searle Ltd. (Oakville, Ont.). Frozen recta were homogenized and deproteinized using $HClO_4$ and assayed as recommended in the kit instructions. Tritium activity was measured by placing samples in 10·0 ml 'Kentfluor' scintillation fluid (Kent Laboratories Ltd., Vancouver, B.C.) and counting in a Nuclear Chicago Isocap 300 liquid scintillation counter, using the channels ratio method of quench correction.

RESULTS

Characteristics of unstimulated recta

The viability of unstimulated recta under short-circuited conditions, as indicated by electrical parameters, is shown in Fig. 1. The open-circuit transepithelial potential difference (PD) declines slowly from an initial value of 32 ± 2 mV (mean ± S.E.M.) following dissection to 12 ± 3 mV after 4 h. The transepithelial membrane resistance (170–220 $\Omega.cm^2$) did not change significantly over the course of the experiment. Short-circuit current (I_{sc}) fell from $6{\cdot}7 \pm 0{\cdot}3$ μequiv.$cm^{-2}.h^{-1}$ initially to $2{\cdot}9 \pm 0{\cdot}3$ μequiv.$cm^{-2}.h^{-1}$ after 2 h. Over the 2nd to 4th h the I_{sc} declined only 0·8 μequiv.$cm^{-2}.h^{-1}$ so that a steady-state condition was approximated. These results are similar to those reported by William *et al.* (1978) using similar *in vitro* preparations. The good agreement between these results is of some interest because Williams *et al.* bathed their preparations in Berridge's complex saline, which contains many organic constituents. Moreover, NaCl concentration was twice as high in the simple saline to maintain osmotic concentration.

Effect of CC homogenate on electrogenic transport

The effects of homogenates of whole CC on rectal transport activity are shown in Fig. 2. One-tenth of a gland pair of CC added to the haemocoel side of the rectum caused the PD to increase from 15 ± 2 mV to 25 ± 2 mV after 80 min. The PD then slowly declined, dropping to 23 ± 2 mV over the next 40 min. Membrane resistance (126–157 $\Omega.cm^2$) showed a slight (7 $\Omega.cm^2$) but significant ($P < 0{\cdot}05$, paired t test) decrease upon the addition of CC homogenate and continued to decline steadily with time. When CC homogenate was added, the I_{sc} began to increase, after a delay of at least 5 min, at a rate of 4·8 μequiv.$cm^{-2}.h^{-2}$ for the next 30 min and continued to rise at a decreasing rate for a further 40 min. Overall, the I_{sc} rose from an unstimulated value of 3·6 to a maximum of 7·0 μequiv.$cm^{-2}.h^{-1}$ at 75 ± 5 min ($n = 22$) after the addition of the homogenate. This peak I_{sc} was maintained or declined very slowly until the end of the experiment at 4 h. Separated storage and glandular lobes of CC both caused stimulation, but not of freshly dissected recta (first 10 min) which already had very high I_{sc} (Fig. 1).

Dose–response relationship for CC homogenate

The response of rectal I_{sc} to increasing amounts of CC homogenate is shown in Fig. 3. There is a good linear relationship between the logarithm of the dosage added and the maximum increase in I_{sc} over the range of 0·002–0·100 pr CC. As little as

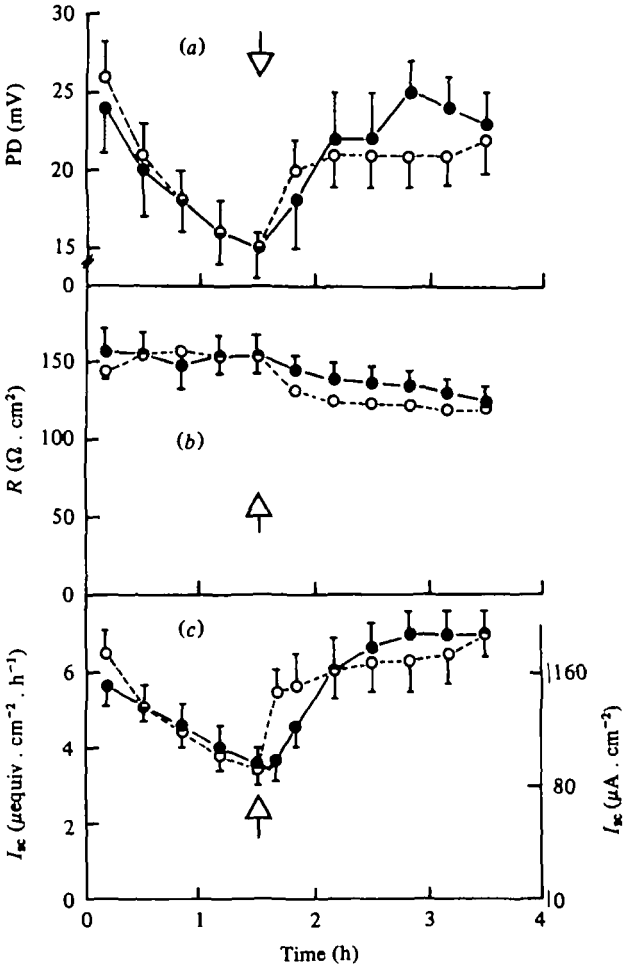

Fig. 2. Effects of CC and cAMP on the electrical parameters of short-circuited recta (mean ±S.E.M.). ●, 0·1 pr CC added at arrow (n = 12); ○, 0·3 mM final concentration cAMP added at arrow (n = 9). (*a*) Transepithelial PD (lumen positive). (*b*) Transepithelial d.c. resistance. (*c*) Short-circuit current, indicating net transfer of anions L → H.

0·005 of a CC pr is sufficient to cause a significant increase in I_{sc} (0·6 ± 0·3 μ-equiv.cm^{-2}.h^{-1}, $P < 0{\cdot}05$).

Individual traces of I_{sc} following exposure of recta to three very high doses of CC homogenate (up to 1·0 pr) are shown in Fig. 4. Although doses in excess of 0·05–0·10 pr CC did not cause further increase in I_{sc}, they did significantly alter the time taken for maximum ΔI_{sc} to fall by one-half ($t_{0.5}$). The $t_{0.5}$ of 52 ± 6 min observed for 0·05 pr CC increased to 88 ± 14 min with 1·0 pr CC. This presumably reflects the greater time that is required for the enzymic destruction of excessive amounts of the CC stimulant. The $t_{0.5}$ observed following stimulation with 0·05 pr CC is similar to that for the initial decline in I_{sc} across unstimulated recta ($t_{0.5}$ of about 1 h, Fig. 1).

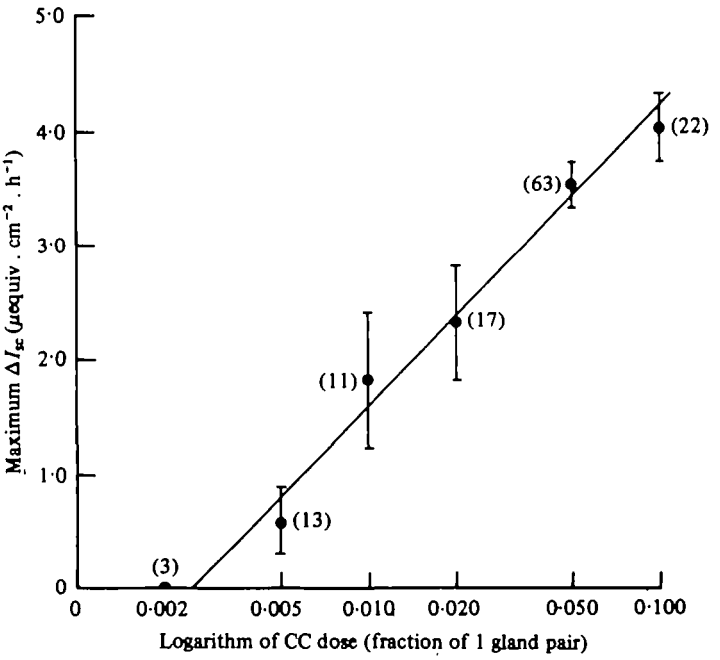

Fig. 3. Maximum increase in I_{sc} across recta (mean ± S.E.M.) with increasing doses of CC. The regression line fitted by 'least squares' method is expressed by $y = 2{\cdot}62 \log x + 6{\cdot}8$; $n = 126$; $r = 0{\cdot}53$; $P < 0{\cdot}05$. Numbers in parentheses indicate n for each point.

A maximum dose of CC usually restores the I_{sc} to approximately the value observed immediately after removing recta from locusts.

Stability of CC homogenate

Boiling CC homogenate for 2–3 min had no measurable effect on its potency (eight trials), although prolonged boiling (> 10 min) destroyed all activity. Homogenates of CC were routinely stored frozen for 4–6 weeks without substantial loss of activity. At room temperature, whole CC in saline still retained about one-half of their original activity after 24 h.

Effects of other tissue homogenates on electrogenic transport

To determine whether the active factor was present exclusively in the CC, the effects of various other tissue homogenates on I_{sc} were tested (Fig. 5). Homogenates of flight muscle caused no stimulation even when used in large quantities (5–10 times the mass of a ventral ganglion), indicating that the active factor was not a general metabolite present in most tissues. Whole brains, ventral ganglia and whole recta, when added in very large amounts, all gave some stimulation but their maximum effect was only equivalent to that caused by 0·005–0·010 pr of CC. These homogenates, unlike CC, were completely inactivated by boiling for 2 min. When allowance is made for the fact that the mass of even the smallest ventral ganglion is 10 times greater

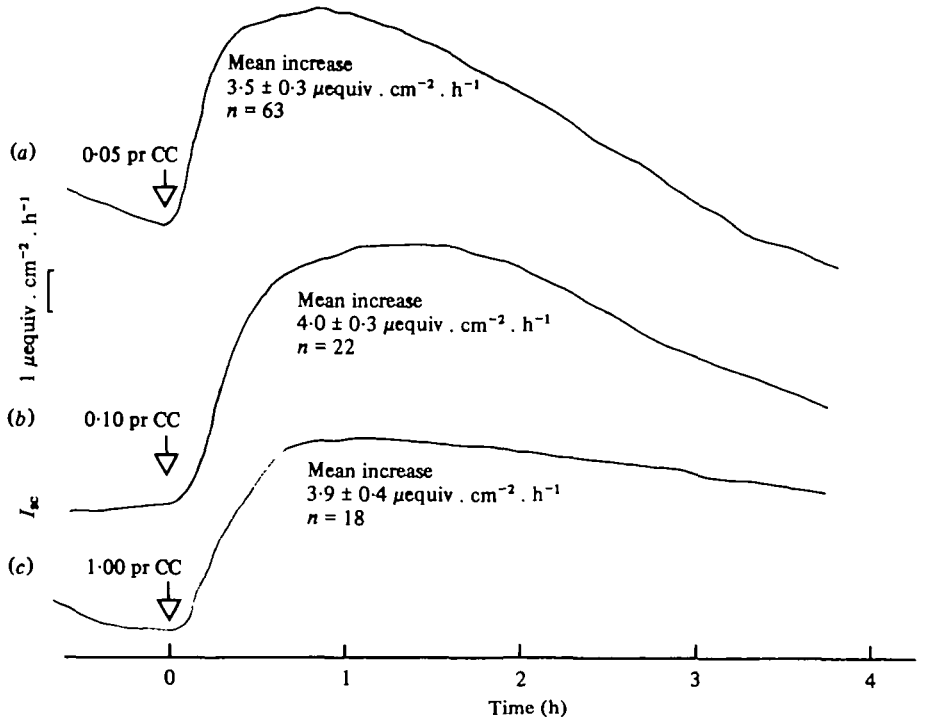

Fig. 4. Typical traces of I_{sc} with time for individual recta stimulated by CC added at arrow. The mean value of ΔI_{sc} for all recta (±S.E.M., number of preparations) at each CC dosage is indicated near the peak of each trace. (*a*) 0·05 pr CC added. (*b*) 0·10 pr CC added. (*c*) 1·00 pr CC added. There is no significant difference among the mean values for ΔI_{sc} in (*a*), (*b*) or (*c*).

than that of 1 pr of CC (about 25 μg), it is clear that I_{sc} stimulating activity is at least 500 times more concentrated in CC than in the other neural tissues which were tested.

The small stimulatory effect of rectal homogenate on I_{sc} might be due to the presence of neurosecretory material in the axon endings which have been observed in this organ. If so, direct electrical stimulation of *in vitro* recta might trigger release of this neurosecretory material. A variety of electrical stimuli (1–5 V, 5 ms duration, 20–50 Hz; 30 s stimulations applied every 1 min for 4–8 min) applied to recta during the steady-state phase had no subsequent effect on I_{sc} (Fig. 5).

Stimulation of electrogenic transport by cAMP

Since the action of peptide hormones is commonly mediated intracellularly by cAMP, we studied the effect of this second messenger on voltage-clamped recta. The results are compared with those for CC stimulation in Fig. 2. Addition of cAMP (final concentration 0·3 mM) to the haemocoel side of *in vitro* preparations caused the PD to increase from 15 ± 1 mV to 22 ± 2 mV after 120 min. There was no measurable lag in the response time and 80% of this increase occurred within the first 20 min. Cyclic-AMP also caused a small but significant drop in membrane resistance (25 Ω . cm², paired *t* test, $P < 0{\cdot}05$). I_{sc} increased within seconds of cAMP application and rose at a rate of 12·6 μequiv . cm⁻² . h⁻² to 80% of its final value within 10 min of applying cAMP.

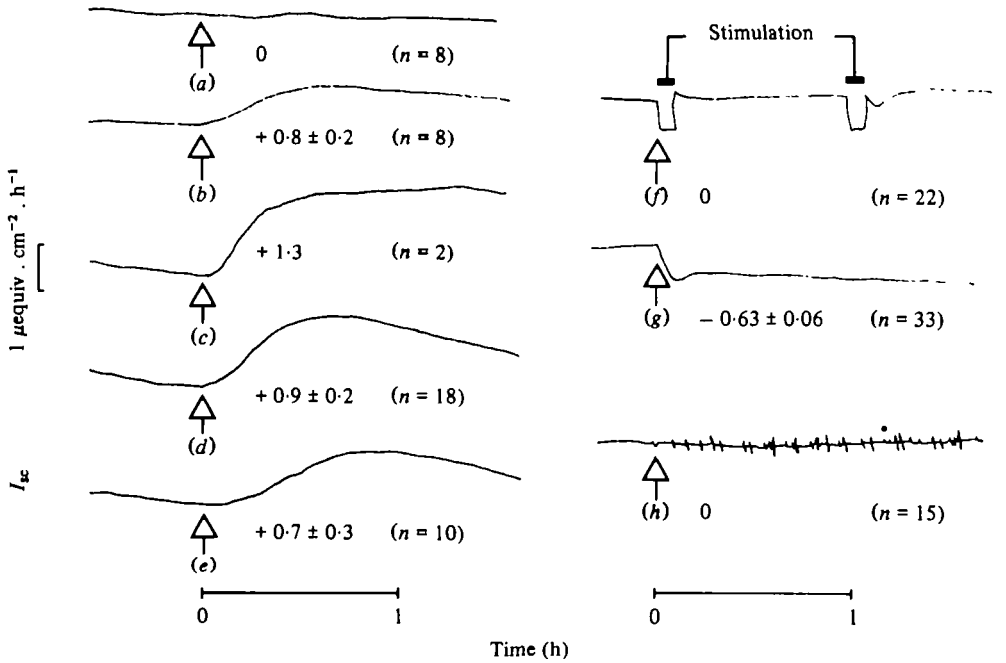

Fig. 5. Effects of tissue homogenates, electrical stimulation and inhibitors on I_{sc}. Typical individual traces are shown as well as mean ± S.E.M. for maximum ΔI_{sc}. Numbers of rectal preparations are shown in parentheses. All substances added at arrows. (*a*) Homogenate of flight muscle. (*b*) 0·5 whole brain. (*c*) 1·0 metathoracic ganglion. (*d*) 0·5 terminal abdominal ganglion. (*e*) 0·5 rectum. (*f*) Electrical stimulation (2·0 V, 20 Hz, 5 ms duration; 30 s stimulations applied every 1 min for 5 min). (*g*) 5×10^{-4} M acetazolamide. (*h*) 10^{-2} M thiocyanate.

As for CC homogenate, the maximum response of rectal I_{sc} to the logarithm of cAMP concentration is linear (Fig. 6). A concentration of 0·005 mM cAMP in the bathing saline on the haemocoel side elicited a measurable response (0·2 ± 0·04 μequiv.cm^{-2}.h^{-1}) and 0·3 mM cAMP caused the same maximal stimulation (ΔI_{sc} of 4·1 μequiv.cm^{-2}.h^{-1}) as 0·1 pr CC.

In summary, cAMP caused a similar maximum increase in I_{sc} as did CC, but these stimulants differed in the lag time of the ressponse following stimulation and the initial rate of rise in I_{sc}.

Effect of CC homogenate on tissue cAMP levels

We obtained some direct evidence that the active factor in CC might normally act on rectal I_{sc} by first elevating intracellular levels of cAMP. When CC homogenate was added to the haemocoel side of everted rectal sacs, the tissue cAMP levels increased 2- to 3-fold within 15 min and remained elevated for at least 1 h (Table 1).

Effects of inhibitors

The work of Williams *et al.* (1978) suggested to us that the increase in I_{sc} upon stimulation might be due to increased Cl^- or H^+/HCO_3^- transport. We therefore

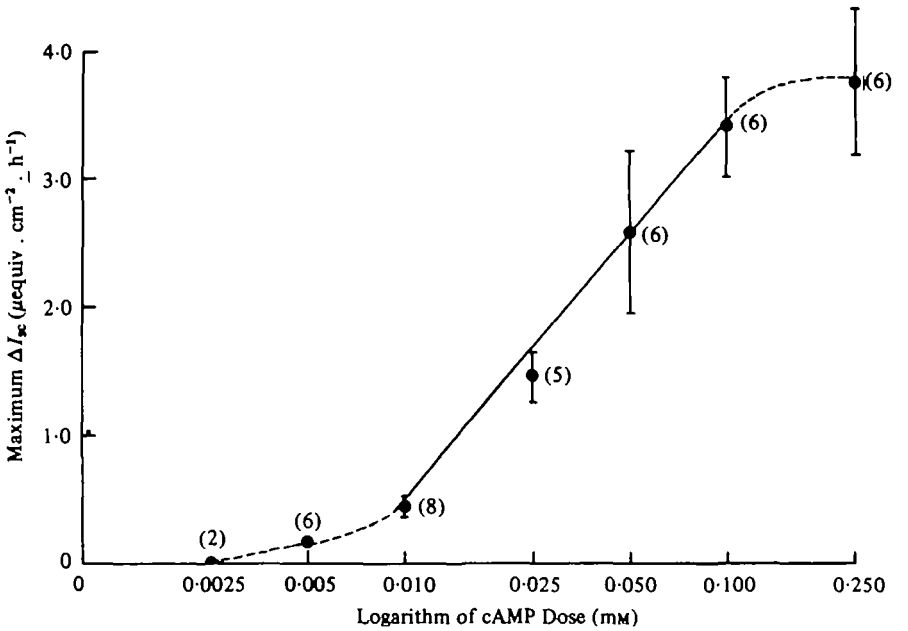

Fig. 6. Maximum response of ΔI_{sc} (mean ± S.E.M.) to increasing doses of cAMP. The regression line by the 'least squares' method for the linear portion of the curve (solid line) is expressed by $y = 2{\cdot}97 \log x + 6{\cdot}4$; $n = 25$; $r = 0{\cdot}84$; $P < 0{\cdot}05$. Numbers in parentheses indicate n for each point.

Table 1. *Effect of CC homogenate added to haemolymph side of everted rectal sacs on tissue levels of cAMP in locust recta*

Treatment	Incubation time (min)	n	cAMP Control of Rectal Tissue (pM.g wet wt ± S.E.M.)
Control (10 μl saline)	60	5	1·3 ± 0·4 (*a*)
CC (10 μl saline + 0·25 pr CC)	15	5	3·1 ± 1·0 (*b*)
CC (10 μl saline + 0·25 pr CC)	30	5	2·5 ± 0·8 (*c*)
CC (10 μl saline + 0·25 pr CC)	60	4	3·1 ± 1·1 (*d*)

(*b*) and (*d*) differ significantly from the control (*a*) at $P < 0{\cdot}05$. (*c*) is not significantly different from the control (*a*).

studied the effects of well-known inhibitors of these transport processes on rectal I_{sc}. Acetazolamide, applied at a concentration of 5×10^{-4} M consistently caused the I_{sc} of unstimulated recta to decrease by 0·63 ± 0·06 μequiv.cm^{-2}.h^{-1} (Fig. 5). The size of this inhibition remained unchanged when recta were stimulated with CC homogenate or cAMP; i.e. this inhibitor did not appear to alter the response to stimulation. Adding a second aliquot to bring the final concentration to 10^{-3} M had no further effect on I_{sc}. Acetazolamide appeared to be equally effective whether added to the lumen or haemocoel side of the preparation.

Thiocyanate, an inhibitor of Cl^- transport in many vertebrate systems, caused no change in the I_{sc} or PD across unstimulated or stimulated recta when added at a final concentration of 10^{-2} M. When acetazolamide and thiocyanate were added simultaneously, the inhibition was the same as that observed with acetazolamide alone.

DISCUSSION

The electrical properties of unstimulated voltage-clamped recta bathed in a simple NaCl saline (Fig. 1) are quantitatively similar to those reported by Williams *et al.* (1978). Our simple saline contains only two organic constituents, glucose and glutamate. Therefore, other organic components in the complex saline used by Williams *et al.* are not necessary to maintain the steady-state I_{sc} or its response to stimulants, at least if Cl^- is present (see Spring & Phillips, 1980*a*).

The main objective of this study was to identify neural or hormonal agents that might normally control electrogenic ion transport processes in the locust rectum. All the neural tissues tested had a slight stimulatory effect on I_{sc}, but the concentration of active factor(s) is at least 500 times higher in the CC than other tissues. That the CC is the major source of a stimulatory agent which is normally released into the haemolymph has been shown more recently by cardiatectomy experiments (Spring & Phillips, 1980*b*).

A slight stimulation of I_{sc} by homogenates of rectal tissue lends some credence to the alternate hypothesis that control might be mediated by neurosecretory nerve endings within the rectal wall (Johnson, 1963, 1966; Gupta & Berridge, 1966; Oschman & Wall, 1969). However, direct electrical stimulation of recta failed to demonstrate local release of a chemical stimulant, perhaps because the nerve endings were already depleted.

Preliminary observations strongly suggest that the active factor in CC homogenate is a peptide. Flight muscle homogenates, even in large quantities, have no effect on I_{sc}, which indicates that the stimulant is not a general metabolite. Known or putative neurotransmitters have been excluded (Spring *et al.* 1978). The action of CC homogenate on rectal I_{sc} is mimicked by cAMP, the second messenger for peptide hormones, and indeed CC causes cAMP levels in rectal tissue to increase substantially (Table 1). The CC factor is water-soluble, suggesting that it is not a steroid, and it is somewhat heat-stable, which implies that it is not a large protein. We have recently semi-purified the active CC factor by gel filtration and found that it has a molecular weight of about 10000 and that it is destroyed by digestion with trypsin (Phillips, Mordue & Spring, in preparation).

Both cAMP and CC homogenates produce linear–log dose-response curves, typical of hormonally controlled processes. However, the external levels of cAMP required for even minimal stimulation are more than one million times greater than the normal levels of this cyclic nucleotide (about 1 pM.mg wet wt^{-1}) in mammalian tissues (Steiner *et al.* 1972; Perkins, 1973) and in unstimulated locust recta (Table 1). It is commonly assumed that the high levels of cAMP required to mimic various hormonal actions reflect the low permeability of the plasma membrane to cyclic nucleotides. It follows that the cAMP contents of tissue homogenates assayed in the present study are several orders of magnitude too low to stimulate rectal I_{sc}.

Although maximum increases in I_{sc} are similar, there are some quantitative differences in the response of recta to cAMP and CC. The initial rate of increase in I_{sc} caused by cAMP is nearly three times greater than for CC homogenate. Moreover, the cAMP acts instantly on recta, whereas there is a 5–15 min lag before I_{sc} responds to CC homogenate, depending on the dose. These differences may reflect the time

required for the CC factor to bind to receptor sites on the rectal epithelia and stimulate intracellular production of cAMP. A similar lag in hormonal stimulation compared to that with externally applied cyclic nucleotides has been frequently observed in other systems (Perkins, 1973; Murad, 1973). Presumably flooding the tissue with cAMP raises the intracellular levels of this nucleotide faster than can be achieved by the adenyl cyclase system alone.

Partial inhibition (25%) of I_{sc} by acetazolamide supports the suggestion by Williams *et al.* (1978) that part of the I_{sc} across unstimulated recta is due to H^+/HCO_3^- transport. However, the failure of acetazolamide to inhibit the increase in I_{sc} caused by cAMP and CC suggests that HCO_3^-/H^+ transport is not stimulated. In our next paper (Spring & Phillips, 1980*a*; also see Spring *et al.* 1978) we report that the increase in I_{sc} following stimulation is due to electrogenic transport of chloride ions. Failure of thiocyanate to decrease rectal I_{sc} (Fig. 5) suggests therefore that Cl^- transport in this tissue is insensitive to this inhibitor.

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