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ACTIVE POTASSIUM TRANSPORT BY THE ISOLATED LEPIDOPTERAN LARVAL MIDGUT: STIMULATION OF NET POTASSIUM FLUX AND ELIMINATION OF THE SLOWER PHASE DECLINE OF THE SHORT-CIRCUIT CURRENT

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SUMMARY

1. Adenosine 3′:5′-cyclic monophosphate (cyclic AMP), Antibiotic A23187, caffeine, cholera toxin, dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP), 5-hydroxytryptamine (5-HT), larval blood, and theophylline were tested for their effects on active potassium transport, as measured by the short-circuit current (SCC), in isolated *Manduca sexta* larval midgut.

2. None of the tested materials affected the short-circuit current during the rapid phase of its usual exponential decline, and only dbcAMP and larval blood affected the short-circuit current during the slower phase of its exponential decline.

3. Dibutyryl-cyclic AMP stimulated an increase in short-circuit current and net potassium flux (measured using ^{42}K). Both unidirectional potassium fluxes increased but there was no effect on the flux ratio or on the rate at which net potassium transport declined with time.

4. A dialysable extract of fifth instar larval blood effected potassium transport in a manner similar to that of dbcAMP, indicating that larval blood contains natural transport-stimulating factors.

5. A dialysable extract of fourth instar larval blood not only stimulated potassium transport but also prevented the slow phase of decline in the short-circuit current. Therefore, in addition to transport-stimulating factors present in fifth instar blood, fourth instar blood appears to contain substances which prevent degeneration of at least the transport related functions of midgut *in vitro*.

6. It is argued that the action of the transport-stimulating factor in larval blood on the electrogenic cation pump in larval midgut cells is mediated by cyclic AMP without involving a change in intracellular calcium ion concentration.

INTRODUCTION

The larval midgut of the tobacco hornworm (*Manduca sexta*) contains a mechanism for the active electrogenic transport of potassium or other alkali metal ions from

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the blood side to the lumen side of the epithelium (Blankemeyer, 1976; Wolfersberger, Harvey & Cioffi, 1982). The midgut potassium pump appears to be identical with the electrogenic cation pump in cells of several other insect epithelia, but quite distinct from the Na^+/K^+ pump of nerve, muscle, and vertebrate epithelial cells (Harvey, 1982). The insect electrogenic cation pump provides the primary driving force for fluid secretion by insect salivary glands and Malpighian tubules (Maddrell, 1977). Fluid secretion by these tissues is stimulated by diuretic hormone (Maddrell, 1962), serotonin (Berridge & Patel, 1968; Maddrell, Pilcher & Gardiner, 1969), and other related substances. The signals generated by interaction of these substances with their receptors at the cell membrane are transduced intracellularly by cyclic AMP and calcium (Berridge, 1980). A large amount of indirect evidence is consistent with the hypothesis that stimulation of fluid secretion by insect epithelia involves a direct effect of cyclic nucleotide, and possibly calcium ion, on the electrogenic cation pump (Maddrell, 1977). If the electrogenic cation pump in the midgut is the same as that in the salivary glands and Malpighian tubules, then one might expect midgut potassium transport to be stimulated by serotonin, diuretic hormone, or related substances. However, there are only two preliminary communications reporting stimulation of active potassium transport in isolated midgut and in both cases the reported stimulants were lipophylic derivatives of cyclic AMP (Smith & Moffett, 1980; Wolfersberger & Giangiacomo, 1980).

The net flux of potassium ion from the blood side to the lumen side of the midgut epithelium (J^K_{BA}) can be conveniently and continuously measured as the short-circuit current (SCC). With isolated midgut, it is consistently observed that after a very brief increase the SCC decreases with time as the sum of two exponential terms (Wood & Moreton, 1978). In the central region of *M. sexta* midgut, the rapid phase of the decline in SCC has a half time of 7 min. Therefore, after approximately 25 min the rapid phase of the decline in the SCC is more than 90% completed and the tissue is in a pseudo-steady-state during which the SCC decreases at a much slower rate ($t_{1/2}$ = 174 min) and there is very close agreement between J^K_{BA} and SCC (Cioffi & Harvey, 1981). Based on morphological evidence, Schultz & Jungreis (1977) concluded that the rapid phase of the decline in SCC was due to massive release of matrix plugs from midgut goblet cells and that the slower phase of the decline in SCC was due to further plug loss plus cell death. These authors proposed that massive plug release is caused by the procedures usually used in mounting the midgut on a chamber and that cell death *in vitro* is due to inadequacies in the usual bathing solution which promote tissue histolysis. However, they were unable to control either phase of the decline in the SCC and did not even speculate on what changes in experimental procedures or bathing solution composition might prevent the decline in SCC. Since Cioffi (1980) has presented very convincing evidence that the goblet cell matrix plugs of Schultz & Jungreis (1977) are actually apocrine secretion droplets from midgut columnar cells, one is left with the hypothesis that loss of cell potassium, preferentially into the lumen side bathing solution by unknown mechanisms, is the most likely cause for the rapid phase of the decline of the SCC (Wood, 1972) and that the slow phase of the decline in SCC is due to tissue degeneration.

In this communication we show that dibutyryl-cyclic AMP not only stimulates the pseudo-steady-state SCC of isolated *M. sexta* midgut in a dose-dependent manner bu

also increases J_{BA}^{K}. We also show that blood from fourth or fifth instar *M. sexta* larvae contains a dialysable factor that increases the pseudo-steady-state SCC of isolated fifth instar midgut. Furthermore, it is shown that dialysable substances in fourth instar larval blood are able to eliminate the slower phase of the decay of the SCC of isolated fifth instar midgut. Some of the results in this paper have previously been presented in abstract form (Wolfersberger & Giangiacomo, 1980).

MATERIALS AND METHODS

Fifth instar larvae of *Manduca sexta* (Lepidoptera, Sphingidae), weighing between 5 g and 7 g, were used for all electrophysiological experiments. These animals were purchased as third instar larvae from Carolina Biological Supply Co. and reared at 26 °C with a 16 h light/8 h dark cycle on a commercial artificial diet. The midgut was isolated as described by Cioffi & Harvey (1981) and mounted on the chamber described by Wood & Moreton (1978), so that only the central region of the epithelium covered the aperture. The average wet weight of the 0·5 cm^2 portion of midgut exposed to bathing solution was 17·8 mg. The bathing solution was composed of 32 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 166 mM-sucrose and 5 mM-Tris HCl, pH 8·3. The bathing solution in each half of the chamber was circulated during all experiments by gas lift pumps operated by oxygen at a flow rate of approximately 5 ml min^{-1}.

The transepithelial potential difference (PD) and short-circuit current (SCC) were measured using the three calomel electrode system described by Wood & Moreton (1978). After measurement of initial PD, each midgut was automatically kept short-circuited by means of a solid state voltage clamp and the SCC was recorded on a potentiometric chart recorder. Unidirectional fluxes of potassium, from the blood side to the lumen side, Φ_{BA}^{K}, and in the reverse direction, Φ_{ab}^{K}, were measured using ^{42}K by the methods of Cioffi & Harvey (1981).

All chemicals were highest purity commercial products. Nucleotides were purchased from Sigma Chemical Company and P-L Biochemicals, Inc. Caffeine, cholera toxin, 5-hydroxytryptamine hydrochloride (5-HT), and theophylline were purchased from Sigma Chemical Co., ^{42}KCl was purchased from New England Nuclear. In order to collect blood from feeding fourth or fifth instar hornworms, the larvae were suspended by their heads, their horns amputated, and the blood was allowed to drain into a small beaker stored on ice. Aliquots of the freshly collected blood were transferred to dialysis tubing sacs (MW cutoff 12 000, A. H. Thomas Co., No. 3787-D12) and dialysed against 100 volumes of distilled deionized water for 24 h at 4 °C. The solution against which the blood had been dialysed was frozen, and the water was removed by freeze drying. The freeze-dried extract was then reconstituted to the same volume as that of the original aliquot of blood using distilled deionized water.

RESULTS

The results of our tests of some prospective effectors of transepithelial potassium transport on the SCC of isolated hornworm midgut are summarized in Table 1. The only commercial compound tested that stimulated the SCC of isolated midgut was N^6, $O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP). Fig. 1 shows that

Table 1. *Effect of prospective effectors of active K^+ transport on the SCC of isolated* Manduca sexta *midgut*

Substance added	Concentration	*Δ SCC*	Concentration range tested
None		$-6{\cdot}8 \pm 1{\cdot}2$ (9)	
Antibiotic A23187	1 μM	$-5{\cdot}3 \pm 0{\cdot}8$ (3)	10^{-6} M to 10^{-4} M
cAMP	10 mM	$-6{\cdot}0 \pm 2{\cdot}3$ (3)	10^{-3} M to 10^{-2} M
Cholera toxin	50 μg ml^{-1}	$-6{\cdot}7 \pm 2{\cdot}0$ (3)	10 μg/ml to 50 μg/ml
dbcAMP	10 mM	$50{\cdot}2 \pm 2{\cdot}8$ (3)	5×10^{-4} M to 3×10^{-2} M
5-HT	1 mM	$-4{\cdot}6 \pm 1{\cdot}5$ (4)	10^{-9} M to 10^{-3} M
Theophylline	50 mM	$-7{\cdot}7 \pm 1{\cdot}0$ (5)	10^{-2} M to 5×10^{-2} M
Caffeine	10 mM	$-7{\cdot}3 \pm 1{\cdot}9$ (3)	
Fourth instar blood extract	1 % (v/v)	$7{\cdot}1 \pm 0{\cdot}7$ (3)	
Fifth instar blood extract	1 % (v/v)	$15{\cdot}7 \pm 3{\cdot}1$ (5)	

All mean changes in SCC are presented in units of $\mu A/cm^{-2}$ min^{-1} ± S.E.M. (N) and are calculated for the 10 min interval immediately following addition of the prospective effector to the chamber. The mean SCC at the time of addition of effector was 550 ± 74 μA cm^{-2}. A concentrated aqueous solution of each compound to be tested, except A23187, was prepared and adjusted to pH 8·3 immediately before use. Concentrated solutions of Antibiotic A23187 were prepared in methyl sulphoxide. Aliquots from the concentrated solutions, of a volume (usually about 1 % of the chamber volume) calculated to give the desired final concentration, were added to the bathing solution on both the blood side and lumen side of the chamber. Addition of up to 0·025 chamber volume of solvent had no effect on SCC. Most substances were tested at several concentrations in addition to the one for which an effect on SCC is listed. In all cases, except that of dbcAMP (Fig. 2), other concentrations of a compound gave results similar to that of the listed concentration of the substance.

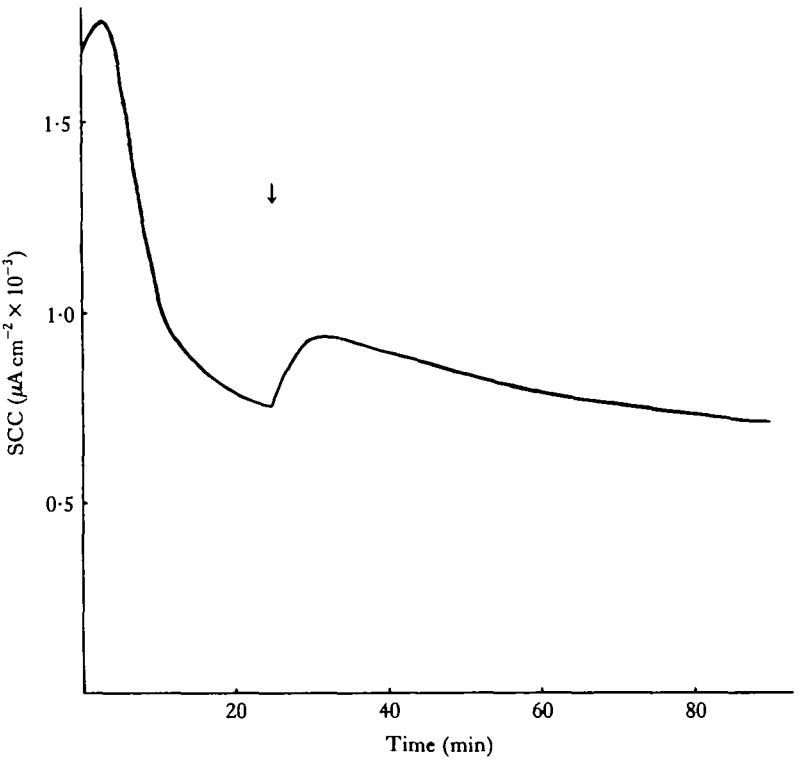

Fig. 1. Effect of dbcAMP on pseudo-steady-state SCC. Tracing of the automatically recorded SCC from a typical experiment in which dbcAMP was added to the chamber at 25 min. The final concentration of dbcAMP in the bathing solution was 2 mM.

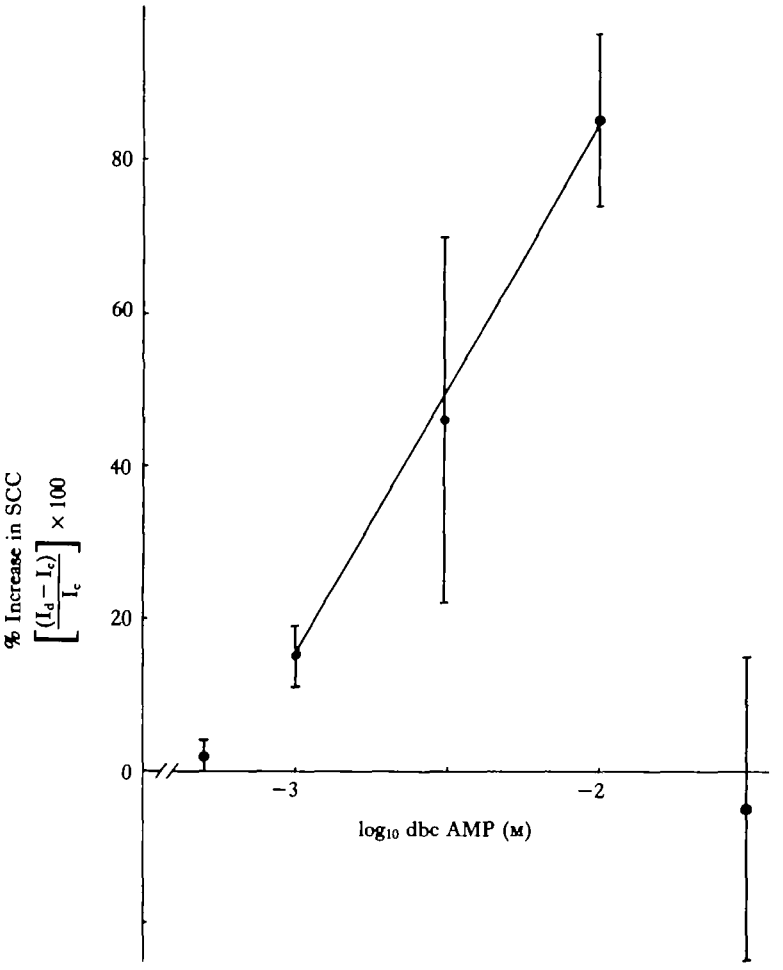

Fig. 2. Increase in SCC vs. log dbcAMP concentration. The percent greater than control increase in SCC, [(maximum SCC after dbcAMP addition – extrapolated control SCC)/extrapolated control SCC] × 100, is linearly proportional to log dbcAMP concentration over the range 10^{-3}M to 10^{-2}M. I_d, maximum SCC after dbcAMP addition; I_c, extrapolated control SCC at the time of maximum SCC. The vertical bars represent ± one S.E.M. ($N = 3$).

when dbcAMP was added to the chamber, during pseudo-steady-state, there was an immediate increase in the SCC. The SCC increased to a maximum value, usually within 10 min, and then resumed a slow exponential decline. The rate of decline of the SCC in dbcAMP-stimulated midguts was the same as that in untreated, control midguts (Table 3). Essentially identical results were obtained whether dbcAMP was added to both sides or only to the blood side bathing solution. However, addition of dbcAMP only to the lumen side was without effect on pseudo-steady-state SCC, and dbcAMP had no discernible effect on SCC if it was added to the bathing solution immediately after mounting the midgut on the chamber. Fig. 2 shows that the maximum amount of stimulation of pseudo-steady-state SCC increased in a linear fashion with the logarithm of dbcAMP concentration for concentrations between 1 mM and 10 mM. 0·5 mM-dbcAMP consistently caused a small increase in SCC, but the average SCC,

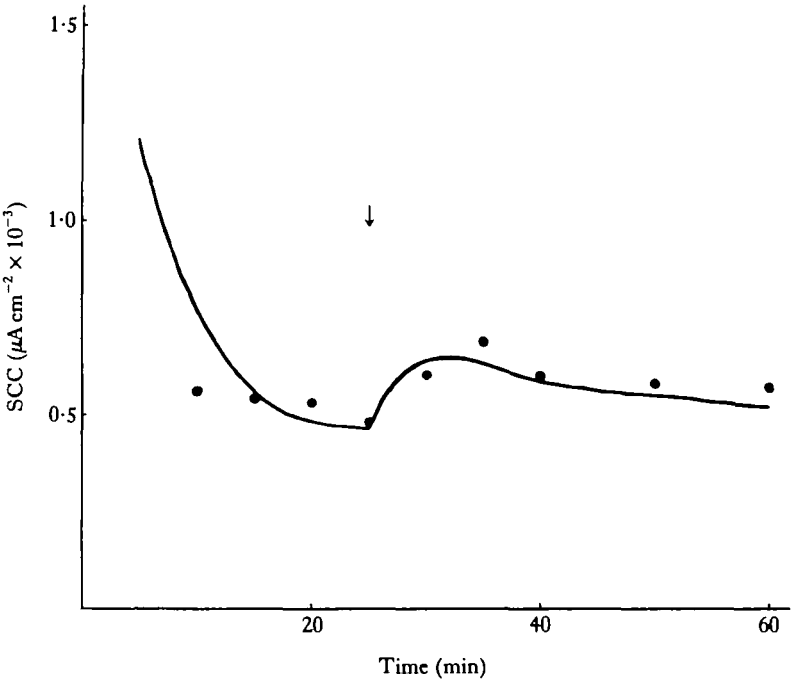

Fig. 3. Effect of dbcAMP on Φ^{K}_{BA}. Data from a typical experiment in which the unidirectional flux of K^+ from the blood side to the lumen side was measured using ^{42}K before and after addition of dbcAMP. At 25 min dbcAMP was added to a final concentration of 2 mM. The solid line is a tracing of the recorded SCC.

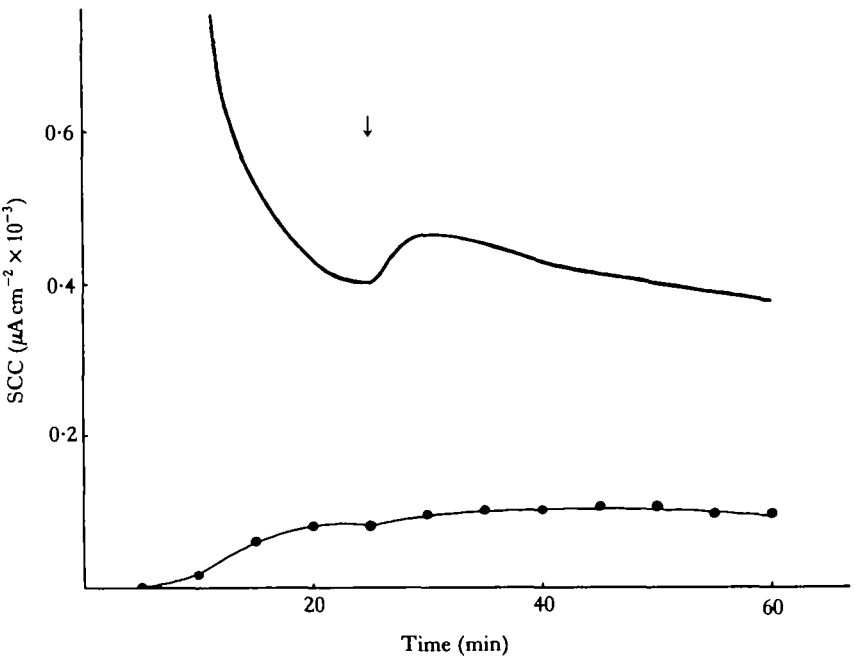

Fig. 4. Effect of dbcAMP on Φ^{K}_{LB}. Data from a typical experiment in which the unidirectional flux of K^+ from lumen side to blood side was measured using ^{42}K before and after addition of dbcAMP. At 25 min dbcAMP was added to a final concentration of 2 mM. The heavy solid line is a tracing of the recorded SCC.

Following addition of dbcAMP to a final concentration of 30 mM, was the same as that predicted in the absence of this compound. dbcAMP caused not only an increase in pseudo-steady-state SCC but also a corresponding increase in Φ^K_{BA} (Fig. 3) and a small increase in Φ^K_{ab} (Fig. 4). These flux experiments showed that dbcAMP stimulates proportional increases in both unidirectional fluxes so that the flux ratio remains unchanged while J^K_{BA} increases (Table 2).

Various concentrations of several other prospective effectors of insect K^+ transport had no significant effect on the pseudo-steady-state SCC of isolated hornworm midgut (Table 1). Our first sample of cyclic AMP from P-L Biochemicals consistently inhibited the decline in short-circuit current during pseudo-steady-state. However, other samples of cyclic AMP from P-L as well as all cyclic AMP samples from Sigma had no effect on SCC. Neither cholera toxin nor Antibiotic A23187 affected the time course of the SCC. If caffeine or theophylline was added to the chamber as an aliquot from a concentrated solution which had been carefully adjusted to pH 8·3, neither of these had a significant effect on SCC. However, direct addition of either of these compounds to the bathing solution in the chamber decreased the pH with a proportional decrease in SCC. For example, addition of theophylline to a final concentration of 20 mM caused the bathing solution pH to drop from 8·3 to 7·7 which was accompanied by a 20 % decrease in the SCC. The effects of adding caffeine or theophylline in combination with cyclic AMP or dbcAMP were no different from the effects of addition of either cyclic nucleotide alone.

The addition of freshly collected larval blood to the solution bathing an isolated and short-circuited midgut resulted in an immediate increase in the SCC. It also caused the stirred bathing solutions to foam vigorously, which led to an imbalance in hydrostatic pressure and damage to the midgut. In order to investigate the effects of components of larval blood on midgut K^+ transport, we prepared protein-free blood extracts by the gentle procedure described in Materials and Methods. Fig. 5 shows that addition of an extract containing the dialysable components of fifth instar larval blood to an isolated and short-circuited midgut caused an immediate increase in the pseudo-steady-state SCC. After reaching a maximum stimulated value, within 10 min after blood extract addition, the SCC declined at the same rate as that in untreated control midguts Table 3. Addition of an extract containing the dialysable components of fourth instar larval blood to an isolated and short-circuited midgut caused an immediate increase in the pseudo-steady-state SCC (Fig. 6). However, the magnitude of the increase and the time course of the decrease in SCC differed from that observed

Table 2. *Effect of dbcAMP on ^{42}K-measured potassium flux across the isolated midgut*

Addition	Φ^K_{BA}	Φ^K_{ab}	J^K_{BA}	SCC	Flux ratio
none	563 ± 39	70 ± 6	493 ± 33	519 ± 58	8·0 ± 0·1
2 mM–dbcAMP	699 ± 93	90 ± 9	609 ± 87	598 ± 69	7·8 ± 0·7

All mean flux values are presented in units of $\mu A\,cm^{-2}$ ± S.E.M. ($N = 3$). Mean SCC values are in the same units ($N = 6$). The flux values in the upper row are for the 5 min interval immediately before addition of dbcAMP and the flux values in the lower row are for the 5 min interval after addition of dbcAMP during which a maximum SCC was recorded. The SCC values are from the midpoint of the 5 min interval for which the flux values are reported.

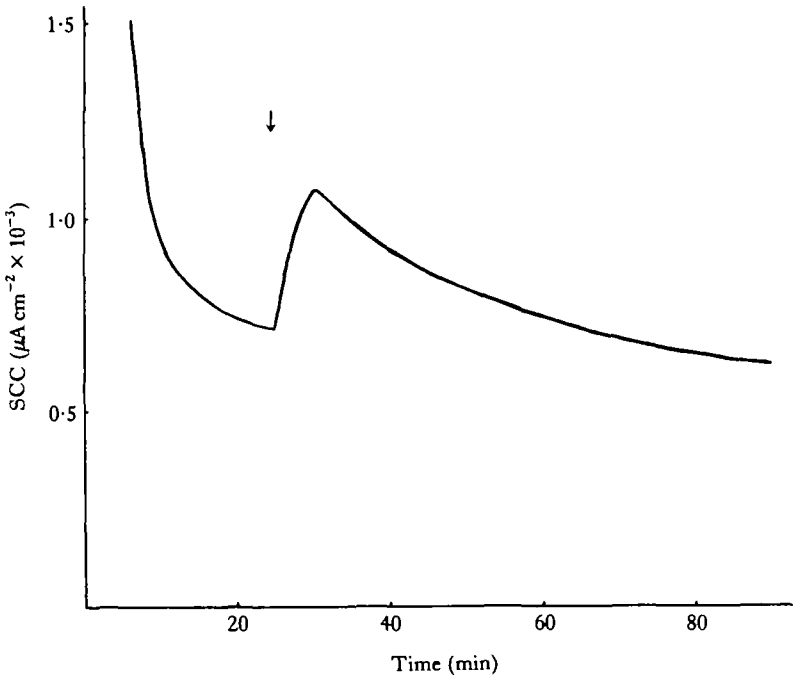

Fig. 5. Effect of fifth instar larval blood extract on SCC. SCC vs. time from a typical experiment in which fifth instar larval blood extract was added to the chamber at 25 min to produce a final concentration of 1% (v/v).

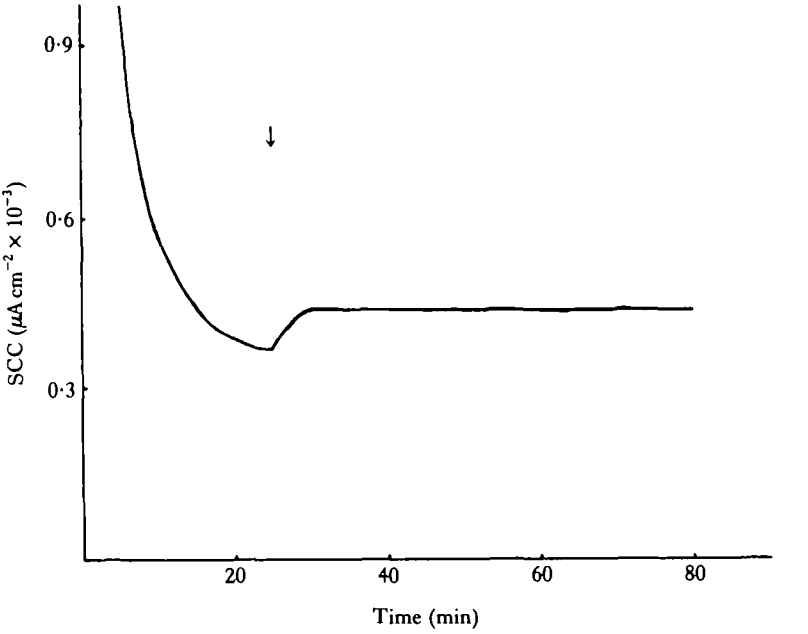

Fig. 6. Effect of fourth instar larval blood extract on SCC. Extract was added at 25 min to produce a final concentration of 1% (v/v).

Table 3. *Effect of dbcAMP and larval blood extract on the rate of decrease in the pseudo-steady-state SCC of isolated midgut*

Addition	Time constant $\times 10^3$
None	$3{\cdot}98 \pm 0{\cdot}42$ (9)
dbcAMP	$4{\cdot}00 \pm 0{\cdot}38$ (12)
Fifth instar blood extract	$3{\cdot}98 \pm 0{\cdot}66$ (7)
Fourth instar blood extract	$0 \pm 0{\cdot}19$ (3)

The time constants for the slow phase exponential decline of the SCC, which resumes immediately after the SCC reaches a maximum stimulated value following addition of effector, are presented in units of min^{-1} ± S.E.M. (*N*).

following addition of fifth instar blood extract. The average maximum increase in SCC following addition of fourth instar larval blood extract was less than 50 % of that caused by the same concentration of fifth instar larval blood extract, but the rate of decline in SCC during a 50 min interval following addition of fourth instar larval blood extract was zero (Table 3).

DISCUSSION

The results presented above clearly show that dbcAMP causes an increase in the rate of pseudo-steady-state potassium transport across the central region of the isolated tobacco hornworm midgut. The cyclic nucleotide causes proportional increases in both unidirectional fluxes of potassium ion so that while the net flux increases the flux ratio remains the same as in untreated midgut. The result that the SCC declines at the same rate in both untreated and dbcAMP treated midguts is consistent with this nucleotide stimulating potassium transport by acting directly on the pump (Berridge, 1980), rather than by stimulating a variety of metabolic processes which might be expected to promote general tissue maintenance and eliminate the slow phase of SCC decline in addition to increasing the rate of potassium transport. The failure of the divalent cation ionophore A23187 (Reed & Lardy, 1972) to stimulate the SCC of isolated midgut (Table 1) argues against the effects of dbcAMP on potassium transport being mediated by calcium ions. The observation that dbcAMP has no effect on potassium transport when added only to the lumen side of the tissue suggests that the apical plasma membrane of midgut cells is either less permeable or contains more phosphodiesterase activity than the basal membrane or both. Finally the result that dbcAMP had no discernible effect on the rapid phase of the decline of the SCC is another argument in favour of a major portion of this current being due to a process other than active transepithelial potassium transport (Cioffi & Harvey, 1981; Wood & Moreton, 1978; Wood, 1972).

The effects of dbcAMP on the midgut are similar to the effects of this compound on the salivary gland (Berridge, 1970, 1977, 1980), even to the extent that the linear portion of the dose versus response curve occurs over the same concentration range in both tissues. However, most of our results with other prospective effectors of active

potassium transport are quite different from those obtained with other insect epithelia. Cyclic AMP, which stimulates the potassium pump in salivary gland and Malpighian tubules (Maddrell, 1980), as well as cholera toxin, which is reported to increase intracellular cyclic AMP concentration (Finkelstein, 1975), had no effect on the potassium pump in the midgut. Methyl xanthines, which are reported to increase intracellular cyclic AMP concentration by inhibiting phosphodiesterase (Butcher & Sutherland, 1962), stimulate salivary gland fluid secretion both alone and synergistically with cyclic nucleotides (Berridge, 1970) but have no effect on midgut potassium transport. In this regard it is interesting to note that theophylline does not stimulate secretion by Malpighian tubules of *Rhodnius* (Maddrell, Pilcher & Gardiner, 1971). Very low concentrations of serotonin stimulate secretion by salivary gland and Malpighian tubules but even millimolar concentrations of this hormone have no effect on midgut potassium transport. All of these negative results are most simply explained by making two reasonable assumptions. (1) The midgut is essentially impermeable to cyclic AMP and methyl xanthines. (2) The plasma membranes of midgut cells lack receptors for 5-hydroxytryptamine and cholera toxin. If these assumptions are correct, there is no reason to deviate from the basic hypothesis, with which our dbcAMP results are completely compatible, that the electrogenic cation pump in the midgut is the same as that responsible for massive Na^+ or K^+ movement in other insect epithelia.

One result of our studies which appears difficult to reconcile with the results of others is our finding that caffeine had no effect on SCC. Smith & Moffett (1980) found that caffeine, at concentrations of 1 mM to 10 mM, inhibited the PD and SCC of isolated tobacco hornworm midgut. Since we assume that these workers were careful to avoid caffeine-induced changes in bathing solution pH, we are left with the possibility that the effects of caffeine on the SCC of the isolated posterior region of hornworm midgut, the region used by Smith & Moffett (D. F. Moffett, personal communication), are different from the effects of caffeine on the central region of hornworm midgut. This explanation is plausible because not only are the potassium transporting goblet cells structurally quite different in these two midgut regions but these two regions of midgut also differ in transport parameters such as potassium pool size, flux ratio, and SCC decay profile (Cioffi & Harvey, 1981).

Comparison of Fig. 5 with Fig. 1 reveals that the effect on pseudo-steady-state SCC of the addition of a dialysable extract of fifth instar larval blood is similar to the effect of addition of dibutyryl-cyclic AMP. In both cases the SCC increases for about 10 min and then declines with the usual exponential time course (Table 3). The final concentration of dialysable blood components required to produce this effect is so low that one can eliminate various ions and metabolites in the extract as causing the effect. Therefore one is led to conclude that larval blood contains a water-soluble transport-stimulating factor. Since attempts to isolate the active substance from blood extracts have been unsuccessful, our knowledge of the chemistry of this factor is quite limited. On the basis of its passing through the dialysis membrane one may conclude that it is a water soluble compound with a molecular weight of less than 12 000 and, based on the failure of 5-HT to stimulate midgut potassium transport, one may conclude that this factor is not closely related in structure to serotonin. The similarity of the tissue response to blood extract and dbcAMP suggests that intracellular transmission

of the signal, generated by interaction of this factor with its receptor on the basal plasma membrane to the potassium pump on the apical plasma membrane, involves cAMP.

Comparison of Fig. 6 with Figs 1 and 5 reveals both similarities and differences between the effects of fourth instar larval blood extract and fifth instar larval blood extract or dbcAMP on the pseudo-steady-state SCC of isolated midgut. In all three cases, the SCC begins to increase immediately after addition of stimulant and reaches a maximum within approximately 10 min. However, the SCC of midguts that have been stimulated by dbcAMP or fifth instar blood extract declines in the usual fashion after reaching the new stimulated level, while the SCC of midguts which have been stimulated by fourth instar blood extract remains steady at the stimulated level and does not decline for at least 50 min (Table 3). These results demonstrate that fourth instar blood contains not only transport-stimulating factors present in fifth instar blood but also other factors not present in fifth instar blood which delay or prevent degeneration of the isolated midgut. Addition of these factors to the bathing medium will allow one to work with an isolated midgut in a true steady-state which will be extremely advantageous for a variety of studies concerning active ion transport by this tissue.

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