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ANTIDIURETIC EFFECTS OF A FACTOR IN BRAIN/CORPORA CARDIACA/CORPORA ALLATA EXTRACT ON FLUID REABSORPTION ACROSS THE CRYPTONEPHRIC COMPLEX OF *MANDUCA SEXTA*

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Summary

Extracts of the brain/corpora cardiaca/corpora allata (Br/CC/CA) complex of *Manduca sexta* larvae elicit an antidiuretic effect, measured by an increase in fluid reabsorption across the cryptonephric complex of larval *M. sexta*. Separation of the extract by reversed-phase liquid chromatography gave two fractions with antidiuretic effects. The more potent of these two factors was further characterized for its effects on the cryptonephric complex. Its antidiuretic effect is not inhibited by bumetanide, a drug that inhibits *M. sexta* diuretic hormone (Mas-DH)-stimulated fluid reabsorption. These data indicate that the mechanism of the antidiuretic effect of the factor is different from that of Mas-DH on the cryptonephric complex. The basal reabsorption of the cryptonephric complex is blocked when treated on the lumen side with bafilomycin A_1, an inhibitor of the H^+-ATPase, or with amiloride, an inhibitor of the H^+/K^+ antiporter. However, the antidiuretic-factor-stimulated fluid reabsorption is not affected by either bafilomycin A_1 or amiloride. The increase in reabsorption triggered by the semi-purified factor can be inhibited by Cl^- channel blockers or by removing Cl^- from the lumen side of the cryptonephric complex. It appears that this factor activates a Cl^- pump associated with the cryptonephric complex. Forskolin mimics the effect of this factor on fluid reabsorption, and the effect of forskolin is not inhibited by bumetanide. A selective and potent inhibitor of protein kinase A, H-89, also inhibits antidiuretic-factor-stimulated fluid reabsorption. Addition of the factor to cryptonephric complexes maintained *in vitro* caused a significant increase in cyclic AMP levels extracted from these tissues compared with values for controls. These data suggest that the antidiuretic effect of the factor in Br/CC/CA extract is mediated by cyclic AMP.

Key words: antidiuretic factor, chloride transport, cyclic AMP, bumetanide, H-89, DPC, DIDS, bafilomycin A_1, amiloride, forskolin, tobacco hornworm, *Manduca sexta*.

Introduction

The composition and volume of haemolymph in insects is largely determined by the excretory system, which is made up of Malpighian tubules and the hindgut (ileum, colon and rectum). The Malpighian tubules produce primary urine which contains most haemolymph solutes. This fluid secretion is driven by a proton pump located on the apical membrane (Wieczorek, 1992) coupled to an H^+/K^+ antiporter (Grinstein and Wieczorek, 1994) which leads to active secretion of salts. The urine then passes into the hindgut, where it is modified to produce a final excreta in the rectum which can be hypo- or hyperosmotic to the haemolymph. The stimulation of Malpighian tubule fluid secretion by synthetic replicates of diuretic factors is now well established [corticotropin releasing factor (CRF)-like peptides (Blackburn et al., 1991; Clark et al., 1998; Clottens et al., 1994; Furuya et al., 1995, 1998; Kataoka et al., 1989; Kay et al., 1991a,b, 1992; Lehmberg et al., 1991), myokinins (Coast and Wheeler, 1990; Hayes et al., 1989, 1994; O'Donnell et al., 1996; Veenstra, 1994), cardioacceleratory peptide 2b (CAP_{2b}) (Davies et al., 1995) and 5-hydroxytryptamine (Clark and Bradley, 1997; Maddrell et al., 1991; Wheeler and Coast, 1990)], but much less is known of the control of fluid reabsorption in the hindgut by antidiuretic factors. The ion transport mechanisms across the hindgut are well characterized in locusts (Phillips et al., 1986); the major process in both the ileum and rectum is an apical electrogenic Cl^- pump, which is stimulated by cyclic AMP (cAMP) and is the primary driving force behind the reabsorption of fluid (Phillips et al., 1986, 1988). However, the only peptides with antidiuretic effects on the hindgut which have been isolated are neuroparsins A and B (Girardie et al., 1989, 1990) and ion transport peptide (ITP) (Audsley et al., 1992), which act on the rectum of *Locusta migratoria* and the ileum of *Schistocerca gregaria*, respectively. The antidiuretic effects of neuroparsins have been questioned (Jeffs and Phillips, 1996; Phillips and Audsley, 1995), whereas the physiological actions of ITP are

well defined: ITP has been shown to promote Cl^-, K^+, Na^+ and fluid reabsorption, and to inhibit H^+ secretion, across locust ileum (Phillips and Audsley, 1995). Phillips et al. (1980) partially purified Cl^- transport stimulating hormone (CTSH), which stimulates KCl and fluid reabsorption across the rectum of *S. gregaria*. CTSH is believed to act *via* cAMP to stimulate an apical Cl^- pump and apical K^+ and basolateral Cl^- conductances (Hanrahan and Phillips, 1983; Phillips et al., 1986); unfortunately, CTSH is extremely labile under acidic conditions, making its isolation by the usual reversed-phase liquid chromatography techniques unfeasible (Phillips and Audsley, 1995).

The control of fluid resorption has been less studied in insects with cryptonephric anatomy, which includes many larval Lepidoptera and all Coleoptera. In the cryptonephric anatomy, the distal ends of the Malpighian tubules do not float freely, but lie very close to the rectum, separated from it by a perirectal space. The structure is ensheathed within the perinephric membrane (of very low water permeability) to form a cryptonephric complex, or rectal complex as defined by Ramsay (1964). Malpighian tubule fluid secretion is a vital part of fluid reabsorption in the cryptonephric complex. Increasing salt and fluid reabsorption from the rectum will promote its recycling through the excretory system, helping to maintain salt and water balance and the clearance of haemolymph waste products. Nicolson (1991) argued that the role of a diuretic hormone in Coleoptera would be largely as a clearance hormone, circulating fluid to moisten the dry food, because of the inability of the coleopteran cryptonephric complex to reabsorb fluid. Audsley et al. (1993) demonstrated that *Manduca sexta* diuretic hormone (Mas-DH) increased fluid uptake from the rectal lumen of the everted rectal sac of *M. sexta*, apparently as a result of stimulation of a bumetanide-sensitive, cAMP-dependent $Na^+/K^+/2Cl^-$ cotransporter (probably located on the basolateral membrane of the cryptonephric tubules), which drew salts from the haemolymph and water from the rectal lumen.

The present study investigates antidiuretic factors present in neuroendocrine tissue of *M. sexta* larvae and whether these factors act on the cryptonephric tubules in a manner similar to Mas-DH or have a direct effect on ion and fluid transport across the rectum.

Materials and methods

Experimental animals

Manduca sexta L. were reared on an artificial diet (Yamamoto, 1969) at 27 °C and 50 % relative humidity with a 16 h:8 h L:D photoperiod.

Chemicals

N-Phenylanthranilic acid (DPC) was purchased from Research Biochemicals Incorporated. Forskolin, bumetanide, bafilomycin A_1, amiloride and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) were purchased from Sigma. *N*-(2-{[3-(4-bromophenyl)-2-propenyl]-amino}-ethyl)-5-iso-quinoline sulphonamide, hydrochloride (H-89) was kindly provided by Dr I. L. O. Buxton (University of Nevada, USA). EDTA was purchased from Aldrich. The protease inhibitors 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride (AEBSF) and E-64 were purchased from Calbiochem. DPC, DIDS, bafilomycin A_1, amiloride, forskolin and bumetanide were prepared as 10× stock solutions in 10 % dimethyl sulphoxide (DMSO) (Fisher Scientific) and diluted in saline for bioassay (final concentration 1 % DMSO in saline). Even at a concentration of 5 % in the saline, DMSO had no effect on fluid transport.

Salines

The saline was based on those described by Chamberlin (1989) for *M. sexta*. Chemicals were added in order as follows: 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 5.8 mmol l^{-1} KOH, 32 mmol l^{-1} KCl, 7.7 mmol l^{-1} tripotassium citrate, 2.8 mmol l^{-1} disodium succinate, 10 mmol l^{-1} glucose, 3.6 mmol l^{-1} alanine, 9.4 mmol l^{-1} glutamine, 12.8 mmol l^{-1} glycine, 9.7 mmol l^{-1} histidine, 5.6 mmol l^{-1} malic acid, 7.4 mmol l^{-1} proline, 8.9 mmol l^{-1} serine, 4.6 mmol l^{-1} threonine, 180 mmol l^{-1} sucrose, 6 mmol l^{-1} Na_2HPO_4 and 10 mmol l^{-1} $NaHCO_3$. The pH was adjusted to 6.7 at room temperature with HNO_3 while the saline was aerated with 95 % O_2/5 % CO_2. Cl^--free saline was made by replacing $MgCl_2$ and $CaCl_2$ with $MgSO_4$ and $CaSO_4$, respectively; KCl was replaced with potassium gluconate.

Bioassay

Everted rectal sacs have been used previously to study fluid transport across the larval cryptonephric complex (Audsley et al., 1993), but details of their preparation were not given. A transverse section of the complex (from Ramsay, 1976) is shown in Fig. 1A. Ramsay (1976) reported that, because of the six muscular attachments of the perinephric membrane to the hypodermis (Fig. 1A), it was not possible to remove the rectal complex without damage. Successful removal may be accomplished as follows: a second-day fifth-instar larva of *M. sexta* was cut open between abdominal segments 6 and 7. Fine pins were used to hold segment 7 and the terminal segment on the dissection dish. The dorsal skin of the larva was cut longitudinally from the anterior of segment 7 to the dorsal horn. The skin was stretched open, and the longitudinal muscles connecting the rectal complex to the hypodermis were cut off carefully, together with the tracheae and associated lipid. The distal ends of the rectal leads of the Malpighian tubules were cut approximately 1 mm from the complex, near the junction with the colon. A piece of polyethylene tubing (i.d. 1.14 mm, o.d. 1.57 mm), with the end slightly flared by heating, was inserted into the anus, and pushed forward into the colon (Fig. 2A). The tissue was ligated at the junction with the colon. The colon was cut anterior to the flare in the tube (Fig. 2A), and the tube was carefully withdrawn from the anus, everting the tissue. Finally, the sac was cut transversely at the anus and ligated. Sacs were maintained in saline at 28 °C and aerated with 95 % O_2/5 % CO_2.

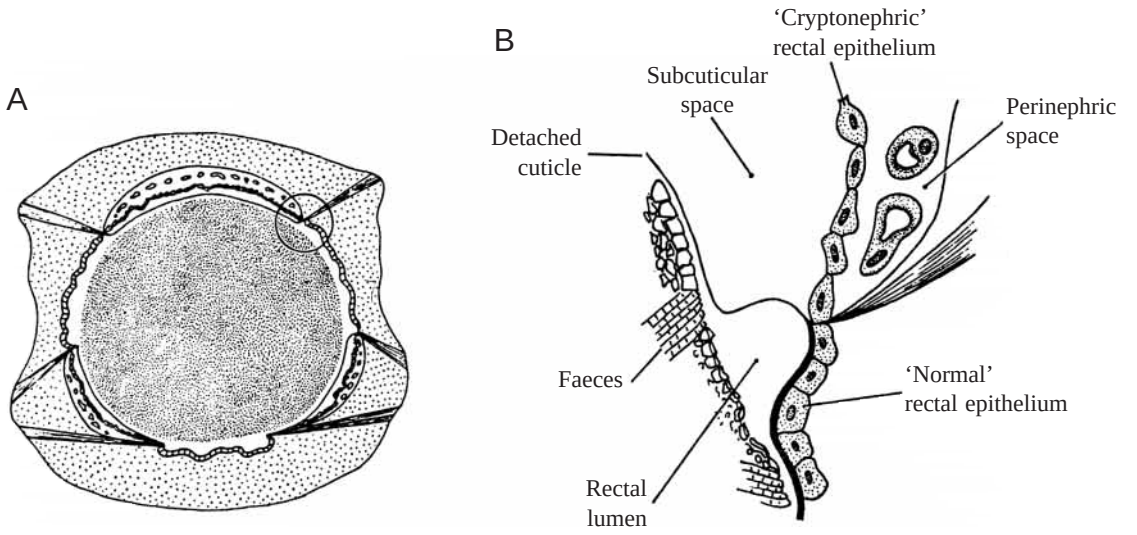

Fig. 1. (A) Diagram of the rectal complex of a lepidopteran larva as seen in transverse section through the posterior end. (B) Detail of encircled region in A. From Ramsay (1976), Fig. 3, reproduced by permission of The Royal Society.

At hourly intervals, weight gain and tissue volume changes were determined by weighing the sacs before and after removal of fluid in the sac. The everted rectal sac plus tube weighed 60–90 mg, and the mass was determined to within 0.2 mg. The true rate of transepithelial fluid movement was determined by correcting for tissue volume changes (Goh and Phillips, 1978). Fluid transport was measured hourly over 5 h, as described by Goh and Phillips (1978). With these preparations, rates are at near steady state after 3 h. Tissue extracts, reversed-phase liquid chromatography (RPLC) fractions and/or chemicals were added to the haemolymph side of the tissue (inside of sac) at the start of the fourth hour, when the tissue had reached a steady state for fluid reabsorption, and rates of fluid reabsorption were compared with those over the previous hour in the same tissue. Thus, each tissue served as its own control on the basis of its rate of reabsorption during the previous hour.

Extraction of tissue and purification

Brain/corpora cardiaca/corpora allata (Br/CC/CA) were dissected from heads of second-day fifth-instar *M. sexta* larvae and stored at −80 °C. When needed, 300 Br/CC/CA were suspended in ice-cold extraction solvent (water, or 20 % or 80 % methanol in water) with protease inhibitors (0.2 mmol l^{-1} AEBSF, 1 mmol l^{-1} EDTA and 10 μmol l^{-1} E-64), then homogenized using a straight-wall tissue grinder. Homogenates were sonicated for 5 min, then centrifuged at 10 000 ***g*** at 4 °C for 5 min. The supernatant was saved for further purification, and the pellet was extracted twice more using the same procedure. Supernatants from each extract were combined for liquid chromatographic purification.

Reversed-phase liquid chromatography separation

Supernatant from an aqueous Br/CC/CA extract was loaded on a 150 mm×4.6 mm PLRP-S (30 nm, 8 μm particle size) reversed-phase column. The column was eluted with a gradient of 2 % to 60 % CH_3CN in 0.1 % trifluoroacetic acid (TFA) over 58 min; fractions were collected manually, generally those associated with spectral peaks absorbing at 220 nm. This was performed on a Perkin Elmer model 410 LC with model 235 UV detector.

Cyclic AMP assay

Measurements of cAMP production by the cryptonephric complex and Malpighian tubules were similar to those of Farndale et al. (1992). Cyclic AMP was extracted from rectal complexes by sonicating in 100 μl of saline supplemented with 0.5 mmol l^{-1} isobutylmethylxanthine (IBMX), heated at 100 °C for 5 min (to precipitate proteins) and centrifuged at 11 000 ***g*** for 20 min. A 50 μl sample of supernatant was taken for cAMP determination. The pellet was then extracted in 3 mol l^{-1} urea for protein determination using a micro BCA assay kit (Pierce). Cyclic AMP released from larval proximal tubules into the saline was quantified directly without extracting the tissue (Audsley et al., 1993).

Results

Solubility of antidiuretic factors in different solvents

The antidiuretic activities of Br/CC/CA extracted in two different solvents (water and 80 % methanol) are shown in Fig. 3. The extracts caused dose-dependent increases in rates of fluid reabsorption across the everted rectal sac. The results of the fluid transport bioassay indicate that 80 % methanol seems to yield the best recovery of antidiuretic factors, especially at low doses. Maximal stimulation was achieved with 0.5 Br/CC/CA equivalents $tissue^{-1}$. Dose-dependent bioassays of 20 % methanol extracts of Br/CC/CA gave results indistinguishable from those of aqueous extracts (data not shown).

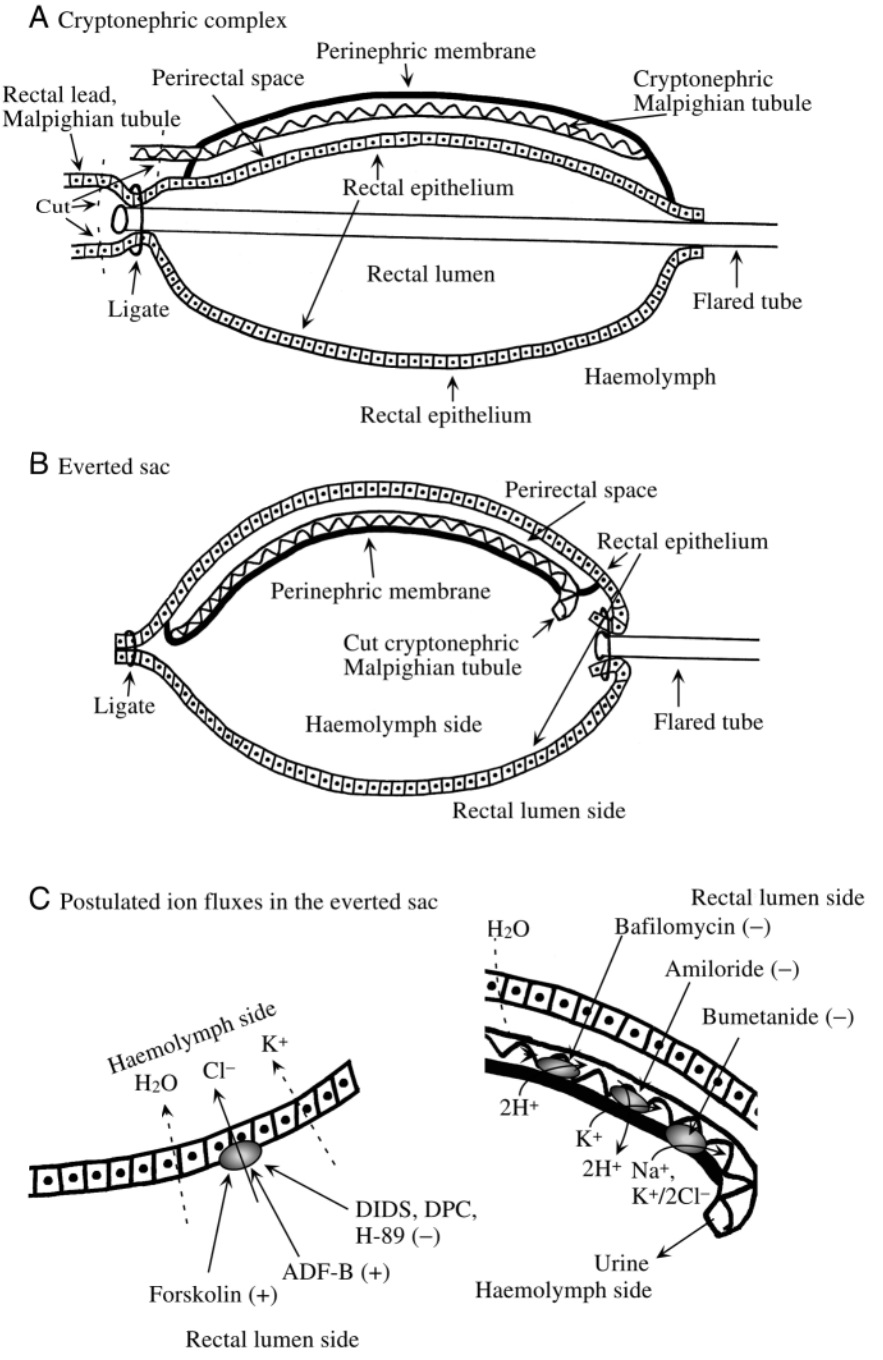

Fig. 2. (A) Diagram of the colon and rectum after insertion of a polyethylene tube. (B) As A, but a ligature has been applied at the junction of the colon and rectum, the tube has been withdrawn until the slight flare stops it, and the rectal leads of the Malpighian tubules have been cut, followed by cutting the anus. The tissue is now everted. (C) As B, but on the left is shown the rectal epithelium in the non-cryptonephric part of the complex depicting the postulated ion transport systems, their stimulators (+) and their inhibitors (–). On the right is shown the cryptonephric part of the complex depicting the postulated ion transport systems and their inhibitors. ADF-B, the antidiuretic factor found in fraction B.

The effect of extract from neuroendocrine tissue of M. sexta *on fluid reabsorption by the cryptonephric complex, and separation of antidiuretic factors by RPLC*

The RPLC profile from the separation of Br/CC/CA extract and the effects of fractions from the separation on rates of fluid transport across larval everted rectal sac are shown in Fig. 4. Two main active fractions were observed, eluting after approximately 40 min (fraction A) and approximately 46 min (fraction B). At a dose of 2.5 Br/CC/CA equivalents tissue^{-1}, fraction A produced a 2.9-fold increase in fluid reabsorption (P<0.02) from 1.74±0.64 to 4.98±0.92 µl h^{-1} tissue^{-1} (mean ± S.E.M., N=5). Similarly, fraction B caused a 3.4-fold increase from 2.64±0.58 to 8.89±0.51 µl h^{-1} tissue^{-1} (P<0.001, means ± S.E.M., N=7). These two active fractions are distinct from Mas-DH, which also stimulates fluid reabsorption by the everted rectal sac (Audsley et al., 1993), because this peptide elutes at 38 min (arrow in Fig. 4) under identical conditions. The concentration of acetonitrile required to elute fraction A was 40–42 % and that required to elute fraction B was 46–48 %. Because fraction B (ADF-B) gave stronger stimulation, we used this fraction to investigate further the mechanism of fluid reabsorption by the complex and to compare its effects with those described for Mas-DH by Audsley et al. (1993).

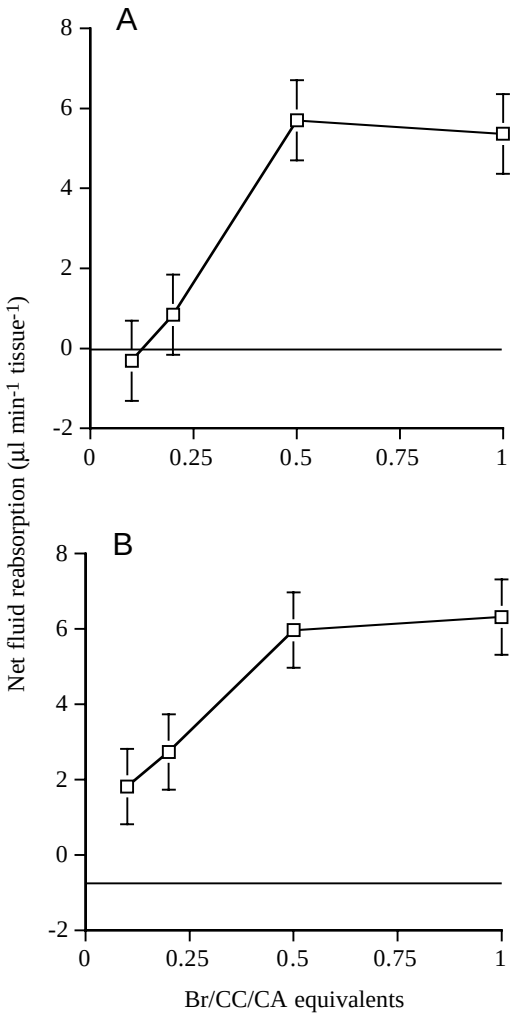

Fig. 3. The dose–response effects of fraction B from semi-purified brain/corpora cardiaca/corpora allata (Br/CC/CA) extracts in stimulating fluid reabsorption across the cryptonephric complex using two different extraction solvents: (A) water; (B) 80 % methanol. Values are means ± S.E.M., *N*=5.

The effect of ADF-B on fluid reabsorption

Mas-DH stimulates fluid reabsorption across the cryptonephric complex *via* a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (Audsley et al., 1993). However, bumetanide (1 mmol l^{-1}) had no effect on ADF-B-stimulated fluid absorption across the everted rectal sac when added either to the haemolymph or to the lumen side of the tissues (Table 1). Bumetanide also did not affect control levels of fluid transport (S. Liao, unpublished observations; Audsley et al., 1993).

Effects of synthetic S. gregaria *ITP on the* M. sexta *everted rectal sac*

Synthetic *S. gregaria* ITP (King et al., 1999) was tested at two concentrations: 1 and 10 µmol l^{-1}. We could not test higher levels because of lack of sufficient sample. At 10 µmol l^{-1}, treated everted rectal sacs showed a reabsorption rate of 3.03±0.57 µl h^{-1} $tissue^{-1}$, whereas controls gave a value of 2.21±0.17 µl h^{-1} $tissue^{-1}$ (means ± S.E.M., *N*=7, *P*>0.05). At 1 µmol l^{-1}, treated everted rectal sac reabsorbed at 5.09±0.63 µl h^{-1} $tissue^{-1}$ compared with controls at 3.68±0.41 µl h^{-1} $tissue^{-1}$ (means ± S.E.M., *N*=9, *P*>0.05). Thus, *S. gregaria* ITP has no statistically significant effect on the rate of fluid reabsorption in *M. sexta*.

Table 1. *The effect of bumetanide on the rate of fraction-B-stimulated fluid absorption across the rectal complex*

	Control (µl h^{-1} $tissue^{-1}$)	Fraction B added (µl h^{-1} $tissue^{-1}$)
1 mmol l^{-1} bumetanide (lumen)	3.53±0.49	9.13±1.20*
1 mmol l^{-1} bumetanide (haemolymph)	3.73±0.74	9.63±0.85*

2.5 Br/CC/CA equivalents of fraction B was added.
*Significantly different from controls (no fraction B) (*P*<0.002).
Values are means ± S.E.M., *N*=6–7.

Cl^--dependence of the effects of ADF-B

Medium free of Cl^- was used to test the ability of Br/CC/CA extracts to stimulate fluid reabsorption in the absence of this ion. ADF-B-stimulated fluid reabsorption was inhibited by removal of Cl^- on the lumen side of the tissue, but not by its removal from the haemolymph side of the tissue (Table 2).

To characterize further this apparent Cl^--dependence, we studied the effect of two Cl^- channel inhibitors. The effects of DPC (0.5 mmol l^{-1}) and DIDS (0.1 mmol l^{-1}) on ADF-B-stimulated fluid reabsorption are shown in Table 3. Both substances completely inhibited the stimulated portion of fluid transport when added to either the haemolymph or lumen side of the tissues, but had no effect on control levels. Addition of either DPC or DIDS in the absence of fraction B did not affect the basal fluid reabsorption.

Effects of inhibitors of the H^+-ATPase and the K^+/H^+ antiporter on basal and ADF-B-stimulated reabsorption of fluid

The basal secretion of insect midgut and Malpighian tubules is driven by a bafilomycin-sensitive vacuolar-type H^+-ATPase (Wieczorek, 1992) coupled to an amiloride-sensitive H^+/K^+ (or H^+/Na^+) antiporter (Grinstein and Wieczorek, 1994).

Table 2. *The effect of Cl^- substitution on the rate of fluid absorption across the everted rectal complex*

	Control (µl h^{-1} $tissue^{-1}$)	Fraction B added (µl h^{-1} $tissue^{-1}$)
Cl^--free in lumen	3.04±0.88	3.49±0.81
Cl^--free in haemolymph	2.86±1.46	9.94±1.13*

2.5 Br/CC/CA equivalents of fraction B was added.
*Mean significantly different from the control (*P*<0.01).
Values are mean ± S.E.M., *N*=5–7.

Table 3. *Effect of Cl^- channel inhibitors (DPC and DIDS) on the rate of fluid absorption by the everted rectal complex*

	Lumen	Haemolymph
DPC (0.5 mmol l^{-1})	1.13	0.93
Fraction B+DPC	1.01	1.26
DIDS (0.1 mmol l^{-1})	0.93	0.95
Fraction B+DIDS	1.26	0.97

2.5 Br/CC/CA equivalents of fraction B was added.

Values shown are stimulation ratios for mean rates of reabsorption in treated tissues (N=4–5) divided by reabsorption values in controls (N=4–5).

Values less than unity indicate lesser reabsorption in treated *versus* control tissues.

In no case was there a statistically significant difference between treated tissues and their corresponding controls.

Bafilomycin A_1 (0.5 μmol l^{-1}) had no effect on basal reabsorption of the larval everted rectal sac of *M. sexta* when assayed on the haemolymph side of this tissue, but it abolished basal reabsorption (P<0.001) when assayed on the lumen side of the tissue (Table 4). This suggests that most of the basal fluid reabsorption across the everted rectal sac preparation is driven by an H^+-ATPase and probably represents the contribution of the Malpighian tubules rather than the rectal epithelium (Fig. 2C). Nonetheless, bafilomycin A_1 had no significant effect on ADF-B-stimulated rates of fluid reabsorption, which increased significantly when this inhibitor was present on either the lumen (P<0.001) or haemolymph (P<0.02) side of the tissue. As can be discerned from these P values, the effect of ADF-B is easier to measure with the basal reabsorption blocked.

Likewise, addition of 1 mmol l^{-1} amiloride to the haemolymph side of the everted rectal sac had no significant effect on the rate of reabsorption. Upon addition to the lumen side, reabsorption was essentially abolished (Table 5), a decrease in reabsorption significant at P<0.01. As with bafilomycin-treated sacs, addition of semi-purified ADF-B to the haemolymph side of amiloride-treated sacs resulted in an

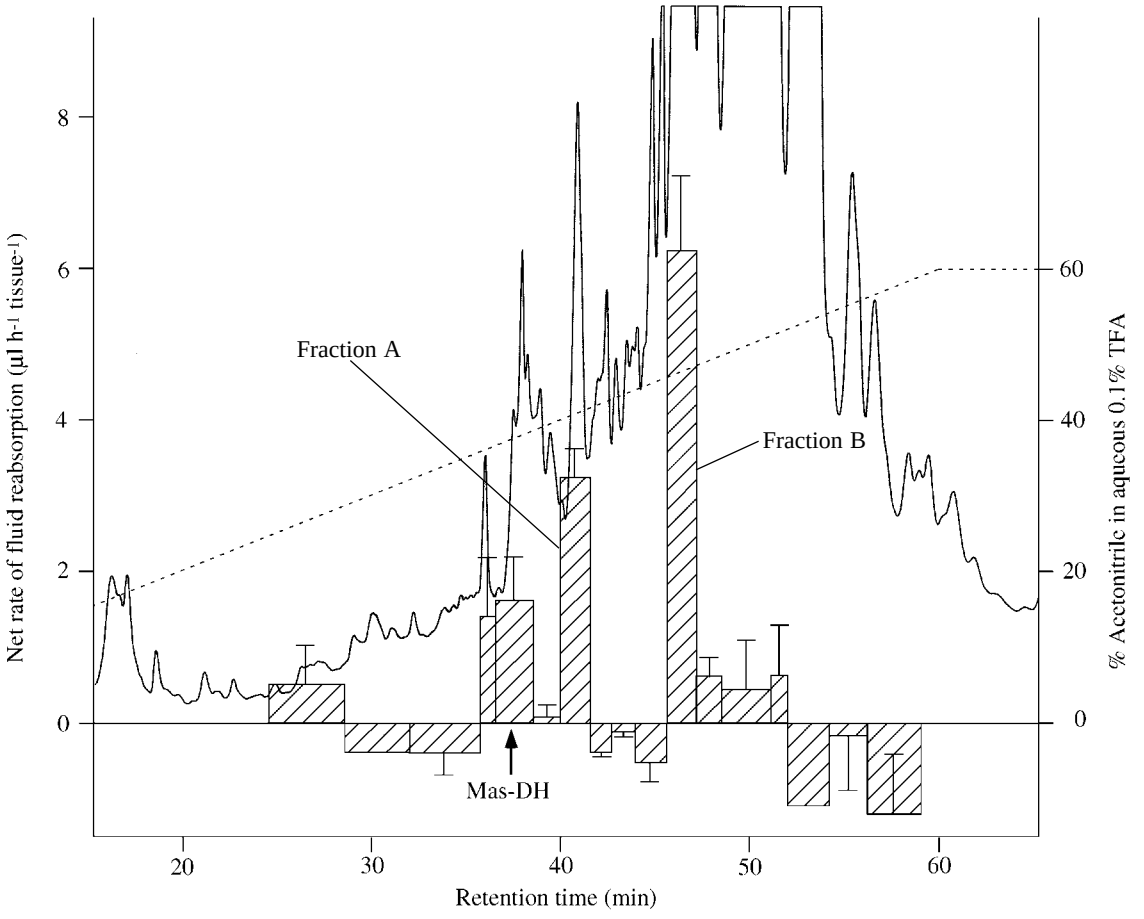

Fig. 4. Purification of an aqueous extract of 300 brain/corpora cardiaca/corpora allata (Br/CC/CA) on a polymeric reversed-phase liquid chromatography column performed as described in Materials and methods. The ultraviolet absorbance trace (solid line) was determined at 220 nm and was 0–1 absorbance units full scale. Fractions were collected manually to include major peaks, rather than on the basis of time. Samples of extracts were dried and assayed as described; the change in the rate of fluid absorption (left axis) is depicted as a histogram. Values are means ± S.E.M., N=4. Several fractions elicited significant increases in the rate of fluid reabsorption; the first of these (indicated by an arrow) occurred at the retention time of *Manduca sexta* diuretic hormone (Mas-DH). Stronger increases in reabsorption were elicited by fractions A and B. The dashed line indicates acetonitrile concentration in the gradient of aqueous 0.1 % trifluoroacetic acid (TFA) (right axis).

Table 4. *The effect of bafilomycin A_1 (0.5 μM) on the rate of fluid absorption across the rectal complex*

	Control (saline) (μl h^{-1} $tissue^{-1}$)	Bafilomycin (μl h^{-1} $tissue^{-1}$)	Bafilomycin+fraction B (μl h^{-1} $tissue^{-1}$)	*N*
Bafilomycin and fraction B on the haemolymph side	2.10±0.62	2.74±0.63	5.94±0.80*	5
Bafilomycin on the lumen side, fraction B on the haemolymph side	1.93±0.16	−1.20±0.70	2.70±0.50‡	4

2.5 Br/CC/CA equivalents of fraction B was added.
Values are mean ± S.E.M.
*Significantly different from the control (normal saline) and bafilomycin (0.5 μmol l^{-1}) control (P<0.02).
‡Significantly different from the bafilomycin A_1 control (P<0.001).

increase in the rate of reabsorption by ⩾3 μl h^{-1} $tissue^{-1}$ (significant at P<0.01). This stimulation in reabsorption is of the same magnitude as that observed in stimulated, untreated sacs (Δreabsorption approximately 3.7 μl h^{-1} $tissue^{-1}$ with bafilomycin, *versus* Δreabsorption approximately 6.2 μl h^{-1} $tissue^{-1}$ in the absence of bafilomycin), whereas if data are calculated as the percentage increase in untreated *versus* treated tissues, the effect on the ratio is far greater with the inhibitor present. These data provide strong evidence that the transport mechanisms driving fluid transport stimulated by ADF-B are independent of those involved in basal reabsorption.

The most thoroughly studied reabsorptive tissue in insects is the locust hindgut. When treated with bafilomycin A_1 at concentrations ten times that used in the present study, only one-third of the active proton secretion into the rectal lumen is blocked (Phillips et al., 1996); basal reabsorption of everted sacs is driven by active Cl^- transport. The dramatic effects of bafilomycin A_1 and amiloride on the *M. sexta* everted rectal sac (Fig. 2C) are highly useful experimentally; basal reabsorption is blocked in even a 1 h incubation (S. Liao and D. A. Schooley, unpublished observations). The basal reabsorption of this complex *in vitro* is probably due to secretion by the Malpighian tubules rather than reabsorption, although how these inhibitors penetrate the Malpighian tubule is hard to explain (see Discussion).

Mediation of the effects of ADF-B by cyclic AMP

Effects of forskolin on fluid absorption

Neither cAMP nor dibutyryl cAMP had any effect on fluid reabsorption across everted rectal sac (results not shown). This finding is puzzling; perhaps the tissue was not completely freed of phosphodiesterases released during dissection of the complex. Forskolin, an activator of adenylyl cyclase, added to the haemolymph side of the tissue at 100 μmol l^{-1}, caused a threefold increase in fluid absorption across the sac (Table 6).

Mas-DH also promotes fluid absorption across the everted rectal sac by a cAMP-dependent mechanism, but stimulates a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (Audsley et al., 1993). Therefore, to determine whether forskolin acts on more than one transport process (which could be stimulated by both ADF-B and Mas-DH), the $Na^+/K^+/2Cl^-$ cotransporter was inhibited by the addition of 1 mmol l^{-1} bumetanide. The results show that forskolin stimulates fluid movement by 2.4-fold (Table 6) in the presence of bumetanide, suggesting that the forskolin-sensitive fluid absorption is largely independent of the $Na^+/K^+/2Cl^-$ cotransporter.

Effects of a protein kinase A inhibitor

A selective and highly potent inhibitor of protein kinase A, H-89 (Chijiwa et al., 1990), was added to everted rectal sac in the presence of ADF-B. H-89 (10 μmol l^{-1}) abolished the ADF-B-stimulated portion of fluid absorption across the everted rectal sac when added to the haemolymph side of the tissue (Table 7), suggesting that this factor is acting *via* cAMP.

Effects of ADF-B on cyclic AMP production

Under control conditions (when no ADF-B was present), the

Table 5. *The effect of 1 mmol l^{-1} amiloride on the rate of fluid absorption by the rectal complex*

	Control (saline) (μl h^{-1} $tissue^{-1}$)	Amiloride (μl h^{-1} $tissue^{-1}$)	Amiloride+fraction B (μl h^{-1} $tissue^{-1}$)
Amiloride and fraction B on the haemolymph side	3.26±0.58	3.42±0.58	6.80±0.73*
Amiloride on lumen side, fraction B on haemolymph side	2.58±0.42	−0.675±0.84	3.04±0.56‡

2.5 Br/CC/CA equivalents of fraction B was added.
Values are mean ± S.E.M., N=5.
1 mmol l^{-1} amiloride on the haemolymph side is not significantly different from control (normal saline).
*Significantly different from control (normal saline) and 1 mmol l^{-1} amiloride (P<0.01).
1 mmol l^{-1} amiloride on the lumen side has a significant effect on the basal fluid absorption (P<0.01).
‡Significantly different from the 1 mmol l^{-1} amiloride value.

Table 6. *The effect of forskolin (added to the haemolymph side of the tissue at 100 µmol l^{-1}), with and without bumetanide (1 mmol l^{-1}), on the rate of fluid absorption across the everted rectal complex*

	Control (saline) (µl h^{-1} tissue^{-1})	Forskolin (µl h^{-1} tissue^{-1})
Without bumetanide	2.68±1.22	7.92±1.06*
With bumetanide	3.83±0.57	9.07±1.20‡

Values are mean ± S.E.M.

*Mean without bumetanide (N=5) significantly different from the control (saline) (P<0.02).

‡Mean with bumetanide (N=6) significantly different from the control (saline) (P<0.001).

amount of cAMP produced by the cryptonephric complex over a 10 min period was 10.1±0.56 pmol mg^{-1} protein (mean ± S.E.M., N=6). ADF-B (5 Br/CC/CA equivalents) caused a significant increase (P<0.001) in cAMP production to 14.1±0.39 pmol mg^{-1} protein by experimental tissues (N=6). However, the same amount of ADF-B had no significant effect (P>0.05) on cAMP production by larval proximal tubules (control 2.86±0.1 pmol tubule^{-1}, treated 1.66± 0.27 pmol tubule^{-1}, N=4, means ± S.E.M.). Under similar conditions, Mas-DH stimulated cAMP production by larval proximal tubules to 20.96±5.11 pmol tubule^{-1} (Audsley et al., 1993).

Discussion

There are fundamental differences in fluid reabsorption between most insect taxa compared with those species possessing the cryptonephric anatomy: lepidopteran larvae and all stages of Coleoptera. In larval Lepidoptera, the rectum has three longitudinally arranged cryptonephric areas, joined by three segments of 'normal' rectal epithelium (Fig. 1; Ramsay, 1976). In contrast, in Coleoptera, the entire rectum is made up of an unbroken cryptonephric complex (Ramsay, 1964). The outer cryptonephric membrane is impermeable to water and devoid of the morphological features of a transporting epithelium (Ramsay, 1964; O'Donnell and Machin, 1991). While both coleopterans and lepidopterans have a high throughput of food, the osmotic problems associated with this are quite different. Many coleopterans live predominantly on a dry diet and must therefore extract virtually all the water from the faeces in the rectum. In contrast, lepidopteran larvae usually live on moist fodder. This led Ramsay (1976) to speculate that the cryptonephric complex evolved in Coleoptera as an adaptation for high recovery of water, but that in Lepidoptera its role is to deal with salt homeostasis. O'Donnell and Machin (1991) postulated that, in coleopterans such as *Tenebrio molitor*, ions enter the complex through the anterior perinephric membrane, accumulating from the haemolymph without the coupled movement of water, then moving into the tubule lumen. Ramsay (1976) proposed that, in lepidopteran larvae, a high osmolarity in the complex was achieved by a combination of 'tidal flow' between the free and cryptonephric tubules and ion uptake by the cryptonephric tubules. In both orders, the cryptonephric tubules aid fluid reabsorption by creating osmotic gradients between the tubules and the rectal lumen.

Table 7. *The effect of H-89 on the rate of fluid absorption across everted rectal complex*

Control (µl h^{-1} tissue^{-1})	Fraction B added (µl h^{-1} tissue^{-1})	Fraction B+H-89 added (µl h^{-1} tissue^{-1})
2.74±0.69	8.27±1.03*	2.56±0.33

2.5 Br/CC/CA equivalents of fraction B was added.

*Mean with fraction B added is significantly different from both other values (P<0.01).

Values are mean ± S.E.M., N=7.

Audsley et al. (1993) found that Mas-DH causes salt transport from the haemolymph side of the *M. sexta* everted rectal sac, with water following from the lumen of the complex. Thus, its net effect on the complex is antidiuretic. Earlier, Nicolson (1991) had argued that the role of a diuretic hormone in Coleoptera would be largely as a clearance hormone because of the inability of the cryptonephric complex to reabsorb fluid. Interestingly, *M. sexta* diuretic peptide II (Mas-DPII) had no effect on the *M. sexta* everted rectal sac at 10 nmol l^{-1} (Audsley et al., 1995), whereas Mas-DH caused a maximal response at 10 nmol l^{-1} with an apparent effective concentration for 50 % response (EC_{50}) in individuals in the range 0.1–0.5 nmol l^{-1}. In contrast, the non-cryptonephric Malpighian tubules of *M. sexta* adults are stimulated by both Mas-DH (Audsley et al., 1993; Kataoka et al., 1989) and Mas-DPII (Audsley et al., 1995; Blackburn et al., 1991; Blackburn and Ma, 1994). Both these peptides also elevate intracellular cAMP levels in the proximal region of larval Malpighian tubule, which is assumed to cause a diuretic response (Audsley et al., 1993, 1995). Hence, in *M. sexta* larvae, Mas-DH has a dual role, a diuretic action on the proximal tubules and an antidiuretic action on the cryptonephric complex, resulting in fluid recycling through the excretory system. In most insect orders, this recycling is due to the combined actions of separate diuretic and antidiuretic peptides and has been proposed as a mechanism to clear toxic and waste products, associated with feeding, from the haemolymph (Phillips, 1983).

In contrast, in most taxa, the Malpighian tubules lie free in the haemolymph, with the blind ends free of the rectum. The best characterized insect reabsorptive tissue is the locust hindgut (Phillips et al., 1986); the major transport process is thought to be an electrogenic Cl^- transporter in the apical membrane of the rectum coupled to a Cl^- conductance on the basal membrane (Phillips et al., 1986). This system is stimulated by cAMP and is the primary driving force behind the reabsorption of fluid. When treated with bafilomycin A_1 at concentrations ten times that used in the present study, only one-third of the active proton secretion into the rectal lumen is blocked (Phillips et al., 1996); basal reabsorption by everted

sacs is driven by active Cl^- transport. The dramatic effects of bafilomycin A_1 and amiloride on the *M. sexta* everted rectal sac are probably due to secretion by the Malpighian tubules rather than to reabsorption (see below).

We have demonstrated the presence in nervous tissue of *M. sexta* of antidiuretic factors (ADFs) which promote fluid reabsorption across the everted rectal sac of larval *M. sexta*, apparently by causing active transport across the normal rectal epithelium (Fig. 1). This composite tissue is a far more complicated experimental system than the well-studied locust rectum (Phillips and Audsley, 1995). The factors are extractable with water or 20 % methanol in water, but 80 % methanol in water appears to give higher recovery (Fig. 3). Because of the generally lower solubility of proteins in 80 % methanol compared with the other solvents, we regard 80 % methanol as the optimal solvent to obtain enriched extracts. These solubility properties are similar to those of ITP from *S. gregaria* (Audsley and Phillips, 1990). Fractionation of an 80 % methanol extract by RPLC showed three fractions causing appreciable reabsorption by the everted rectal sac (Fig. 4); the first of these is coincident with the retention time of Mas-DH, while the two others elute more slowly; we term them ADF-A and ADF-B. A detailed study of the more potent, or more abundant, of these two factors, ADF-B (fraction B), has revealed a number of its characteristics. We also tested the effects of synthetic *S. gregaria* ITP (King et al., 1999) at up to $10\,\mu mol\,l^{-1}$ in our assay; no statistically significant effect was seen.

Bumetanide (which inhibits $Na^+/K^+/2Cl^-$ cotransport) reduces fluid reabsorption induced by Mas-DH to steady-state levels (Audsley et al., 1993). We found it to have no effect on *M. sexta* ADF-B-stimulated fluid transport, providing evidence that these two factors act on different ion transport systems.

We studied the effects of *M. sexta* ADF-B on rectal ion transport processes. The effect of this factor is independent of the basal reabsorption of the everted rectal sac preparation *in vitro* (see below). Removal of Cl^- from the saline on the lumen, but not the haemolymph, side of the tissue abolishes the ability of ADF to stimulate fluid reabsorption across the everted rectal sac. This suggests that this factor acts on Cl^- reabsorption from the lumen, most likely on the 'normal' rectal epithelium. In contrast, Mas-DH-stimulated fluid absorption across this complex is abolished by removal of Cl^-, Na^+ or K^+ from the haemolymph side of the tissue only (Audsley et al., 1993).

DPC, a Cl^- channel blocker, and DIDS, an anion channel blocker (Cabantchik and Greger, 1992), both abolish *M. sexta* ADF-B-stimulated fluid movement across the cryptonephric complex of *M. sexta*, but the effects of these inhibitors on Mas-DH action have not been tested. DPC also abolishes the cAMP-stimulated Cl^--dependent short-circuit current across both locust rectum and ileum (N. Audsley, unpublished observation). DPC is generally regarded primarily as a Cl^- channel blocker, but at high concentrations it affects other Cl^- conductive pathways (Cabantchik and Greger, 1992). DIDS and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) belong to the disulphonic stilbene family and DPC to the arylaminobenzoate family. These inhibitors are known to act from both sides of the membrane in other systems, although effects may differ on the lumen compared with the haemolymph side (Cabantchik and Greger, 1992). Curiously, SITS was reported to have no effect on Cl^- transport across locust rectum (Hanrahan and Phillips, 1983); this negative result must be interpreted with caution because their saline contained 'amino acids at their natural levels' (Hanrahan and Phillips, 1983). Both SITS and DIDS (aromatic thiocyanates) can react with buffer constituents containing primary amino groups (such as amino acids and Tris) with a concomitant loss of inhibitory potency (Cabantchik and Greger, 1992). While our saline contains amino acids, DIDS was added to the saline immediately before experiments; DIDS-containing salines were discarded after use.

Similarly, inhibitors of the vacuolar H^+-ATPase (bafilomycin A_1) and an H^+/K^+-antiporter (amiloride) have no effect on the antidiuretic-stimulated reabsorption, but abolish basal fluid absorption of the complex when added to the lumen side *in vitro*. In the locust rectum, Phillips et al. (1996) have demonstrated the existence of a bafilomycin-sensitive vacuolar ATPase, but its rate of proton pumping is 10–15 % of that of Cl^- pumping. Amiloride abolishes reabsorption caused by Mas-DH (S. Liao, unpublished observations), but has no effect on ADF-B-stimulated rates of fluid reabsorption. These data suggest that bafilomycin and amiloride act on the cryptonephric tubules rather than the rectum. It is possible that their actions may be observed only when applied to the lumen because the rectal and Malpighian tubule epithelia are permeable to these drugs, whereas the perinephric membrane is not (restricting action from the haemolymph side). The use of these inhibitors strongly suggests (1) that the basal reabsorption of the everted rectal sac is due to active salt transport by the cryptonephric Malpighian tubules, and (2) that ADF-B-stimulated fluid reabsorption acts *via* a fundamentally different transport mechanism. Although treatment of rectal sacs with both bafilomycin A_1 (Table 4) and amiloride (Table 5) appears to give a slight reversal in the direction of fluid flow, these values were not significantly different from zero.

M. sexta ADF-B causes a significant increase in cAMP production by the cryptonephric complex, presumably by increasing intracellular levels within the rectal epithelium. However, Audsley et al. (1993) also report that Mas-DH increases levels of this cyclic nucleotide produced by the cryptonephric complex, probably by increasing amounts in the cryptonephric tubules. Curiously, exogenous cAMP and dibutyryl cAMP have no effect on fluid reabsorption across the cryptonephric complex, yet cAMP stimulates fluid reabsorption across the rectum and Malpighian tubules of locusts (Morgan and Mordue, 1985; Proux et al., 1984). We incubated everted rectal sac with forskolin, which activates adenylyl cyclase, to increase intracellular cAMP levels and found stimulation of fluid reabsorption. To rule out the possibility that this fluid transport was due to stimulation of the $Na^+/K^+/2Cl^-$ cotransporter in the cryptonephric tubules (mimicking Mas-DH action), we added both forskolin and

bumetanide to everted rectal sacs, yet fluid transport persisted. Thus, forskolin must act predominantly on a transport process other than the $Na^+/K^+/2Cl^-$ cotransporter, probably Cl^- transport across the rectal epithelium, and mimics ADF-B action. In addition, H-89, a selective and potent inhibitor of protein kinase A (Chijiwa et al., 1990), inhibits fluid reabsorption otherwise stimulated by *M. sexta* ADF-B. These observations suggest that the effect of ADF-B is mediated *via* cAMP and cAMP-dependent protein kinase A.

CTSH from *S. gregaria* corpora cardiaca, which stimulates KCl and fluid reabsorption across locust rectum, was partially purified by gel filtration chromatography and was estimated to have a relative molecular mass of approximately 8000 Da. Hanrahan and Phillips (1984) proposed that CTSH acts *via* cAMP to stimulate the apical Cl^- pump and apical K^+ and basolateral Cl^- conductances. Proux et al. (1984) showed that CTSH-stimulated fluid transport across the everted rectal sac is Cl^--dependent. Our results reveal some similarities between *M. sexta* ADF-B and CTSH: both are extracted best in 80 % methanol, both are thought to act *via* cAMP to stimulate fluid reabsorption across the rectum, and the mechanism of both is Cl^--dependent. There are also great similarities with the action of ITP across locust ileum (Phillips and Audsley, 1995).

On the basis of our results, we conclude that ADF-B is distinct from Mas-DH; its actions appear to involve stimulating fluid reabsorption by stimulating active Cl^- transport across the rectal epithelium within the cryptonephric complex. This fluid probably enters the haemolymph *via* the 'normal' rectal epithelium, which is not embedded in the cryptonephric segments of the rectum (Fig. 1). Moffett and Cummings (1994) postulated that fluid reabsorption in *M. sexta* occurs not only in the cryptonephric complex but also in the ileum, because the alkaline fluid leaving the midgut is neutralized in the ileum. However, Reynolds and Bellward (1989) found no evidence supporting a reabsorptive role for the ileum in *M. sexta*, and N. Audsley (unpublished observations) found low rates of basal reabsorption by everted ileal sacs in *M. sexta*; these low rates were not stimulated by Br/CC/CA extracts.

If CTSH and *M. sexta* ADF-B are similar peptides, they may stimulate Cl^- reabsorption by a similar mechanism, namely by increasing intracellular cAMP levels to activate an electrogenic Cl^- transporter on one membrane and a Cl^- conductance on the opposing membrane of the rectal epithelium. However, because the structure of the cryptonephric complex is not amenable to the voltage-clamp technique used to study the effects of CTSH on locust rectum, this must be investigated using ion-sensitive microelectrodes.

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