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SYNERGISM OF HORMONES CONTROLLING EPITHELIAL FLUID TRANSPORT IN AN INSECT

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Summary

Forskolin stimulates rapid fluid secretion by the Malpighian tubules of *Rhodnius prolixus* at concentrations above 5×10^{-6} mol l^{-1}. In the presence of a threshold concentration of forskolin, the tubules are 30–50 times more sensitive to 5-hydroxytryptamine (5-HT) than in its absence. Similar synergism is seen between 5-HT and extracts of the mesothoracic ganglionic mass (which is rich in the peptide diuretic hormone, DH) and between 5-HT and samples of haemolymph, also rich in peptide DH, from fed insects 1–2h after feeding. The dose–response curves for mixtures of forskolin and 5-HT and of peptide DH and 5-HT are all very steep, approximately five times steeper than for any one stimulant alone.

Forskolin, 5-HT and extracts of the ganglionic mass all stimulated adenylate cyclase from broken membrane preparations from the Malpighian tubules in a dose-dependent manner and at doses similar to those required to stimulate fluid secretion by intact tubules. Mixtures of ganglionic extract and 5-HT stimulated adenylate cyclase activity in a synergistic fashion.

Injections into fifth-instar *Rhodnius*, 24h before feeding, of 5,7-dihydroxytryptamine, which is known to block or reduce 5-HT release, caused delays in the onset of the consequent diuresis or prevented it altogether. This is consistent with the proposal that the rapid onset of diuresis after feeding is caused by the simultaneous release of 5-HT and peptide DH acting synergistically.

Introduction

Rhodnius prolixus is a blood-sucking insect in which there is a rapid elimination of a large volume of fluid, consisting largely of a hypo-osmotic NaCl solution, after each of its large blood meals (Wigglesworth, 1931; Ramsay, 1952; Maddrell, 1964). This diuresis is in part stimulated by a peptide diuretic hormone (DH) that accelerates fluid secretion by the Malpighian tubules by up to a thousand times (Maddrell, 1963; Aston and White, 1974). We have recently shown that 5-HT (5-hydroxytryptamine; serotonin), long known to stimulate *Rhodnius*' tubules *in vitro* (Maddrell *et al.* 1969, 1971), also acts as a diuretic

Key words: synergism, insect diuretic hormones, serotonin, 5-HT, forskolin, ketanserin, cyclic AMP, adenylate cyclase, *Rhodnius prolixus*, Malpighian tubules, epithelia.

hormone after a meal in this species (Maddrell *et al.* 1991). We suggested that the two diuretic hormones might act synergistically to stimulate fluid secretion by the Malpighian tubules. Such an idea was not new, for Barrett and Orchard (1990) had earlier shown that 5-HT would cause an increase in intracellular levels of cyclic AMP of tubules from both larval and adult *Rhodnius*, leading them also to the idea that 5-HT and the peptide hormone might act synergistically on the tubules.

In the present paper, we provide evidence that the two hormones, peptide DH and 5-HT, act synergistically to stimulate fluid secretion by the Malpighian tubules, that the dose–response relationship for the combination of the two is markedly steeper than for a single stimulating substance and that both hormones cooperate synergistically to activate adenylate cyclase in broken membranes from tubule cells. In addition, we show that interfering specifically with the release of 5-HT after a meal delays or entirely prevents the normal diuresis.

Materials and methods

The insects used were *Rhodnius prolixus* Stål (Hemiptera) kept at 27°C in laboratory culture at the Department of Zoology, Cambridge, UK. Experiments were performed with third- and fifth-stage (juvenile) insects.

Malpighian tubules were dissected from insects under saline using a binocular microscope and isolated in drops of the saline under liquid paraffin as described earlier (Maddrell *et al.* 1988). The standard saline initially used had the following composition ($mmol\,l^{-1}$): NaCl, 143; KCl, 8.6; $CaCl_2$, 2.0; $MgCl_2$, 8.5; Hepes, 8.6; glucose, 34; pH adjusted to 7.0 with NaOH. In later experiments, a version improved to include bicarbonate and phosphate was used; it had the following composition ($mmol\,l^{-1}$): NaCl, 129; KCl, 8.6; $CaCl_2$, 2.0; $MgCl_2$, 8.5; $NaHCO_3$, 10.2; NaH_2PO_4, 4.3; Hepes, 8.6; glucose, 20; pH adjusted to 7.0 with NaOH. The importance of working with bicarbonate-based media is emphasised by Thomas (1989), who acidly observed that ‘he who works with bicarbonate-free media risks studying cellular and molecular pathology rather than physiology’.

5-Hydroxytryptamine and 5,7-dihydroxytryptamine were obtained from Sigma; ketanserin tartrate was from Cambio and GDPβS from Boehringer. 1,9-Dideoxyforskolin was kindly given to us by Dr P. D. Evans.

Extracts of mesothoracic ganglionic masses were made from ganglia previously dissected out and stored at −20°C. A suitable number of ganglia were placed in a glass homogeniser, disrupted, diluted with saline, sonicated for 30s and held on ice until used, usually within 15min. Where the extracts were used to stimulate adenylate cyclase from Malpighian tubule membranes, they were first heat treated at 100°C for 2–3min to destroy the intrinsic adenylate cyclase in the masses.

For determinations of adenylate cyclase in broken membranes from Malpighian tubules, 80 tubules from fifth-instar insects were suspended in $2cm^3$ of ice-cold buffer (154$mmol\,l^{-1}$ NaCl, 1$mmol\,l^{-1}$ EDTA, 10$mmol\,l^{-1}$ Tris/HCl, pH7.4). They were ruptured by 40 strokes of a Teflon–glass homogeniser rotating at 1350$revs\,min^{-1}$. The homogenate was diluted in the same buffer to $25cm^3$, centrifuged at 70000g (maximum)

for 25min and the pellet resuspended in 1mmol l^{-1} dithiothreitol (DTT), 25mmol l^{-1} Tris/HCl, pH7.4 to about 200 μg cm^{-3} protein (Lowry *et al.* 1951) and kept on ice for up to 2h until used. Adenylate cyclase activity was estimated, essentially as described by Knowles and Farndale (1988), by incubating about 10 μg protein per sample in 1 mmol l^{-1} DTT, 25mmol l^{-1} Tris/HCl, pH7.4 with 10mmol l^{-1} Mg^{2+}, 10 μmol l^{-1} guanylyl (β,γ-imido) diphosphate (pNHppG), 100 μmol l^{-1} ATP, 500 μmol l^{-1} cyclic AMP (all from Boehringer) and 10^6 disints min^{-1} [α-^{32}P]ATP (ICN) with a creatine phosphate/creatine kinase ATP-regenerating system. The total assay volume was made up to 100 μl with buffer after adding the required amounts of agonist and/or antagonist. After 20min at 30°C, the reaction was terminated and ^{32}P-labelled cyclic AMP separated (Saloman *et al.* 1974) and determined by liquid scintillation counting.

Results

Synergism of 5-HT and forskolin

Earlier studies reported an elevation of cyclic AMP levels in the cells of *Rhodnius*' tubules caused by both treatment with material (presumed to be peptide DH) released from the insect's mesothoracic ganglionic mass by exposure to K^+-rich saline (Aston, 1975) and by treatment with 5-HT (Barrett and Orchard, 1990). Cyclic AMP levels were also found to increase in fluid secreted by tubules stimulated with 5-HT (Montoreano *et al.* 1990). It seems likely that Aston's experiments with K^+-rich saline would also have released 5-HT, but, on the face of it, these data allow the idea that both diuretic hormones might activate adenylate cyclase as one of their actions; Barrett and Orchard (1990) did not doubt that the peptide hormone acted through cyclic AMP.

To test the possibility that two stimulants acting *via* adenylate cyclase might cooperate synergistically, we examined the effects of treating Malpighian tubules with forskolin by itself and in combination with 5-HT. Forskolin is known to stimulate adenylate cyclase in many vertebrate systems and it also frequently potentiates the action of hormones which stimulate that enzyme (Seamon and Daly, 1981). Forskolin was able to stimulate fluid secretion by *Rhodnius* Malpighian tubules (Fig. 1); the dose required to give half-maximal stimulation was about 2.5×10^{-5} mol l^{-1}. This suggests that forskolin might act by stimulation of adenylate cyclase in *Rhodnius* as it does in other systems. However, in other systems, forskolin may act through other routes (Laurenza *et al.* 1989; Baxter and Byrne, 1990). To eliminate this possibility, we treated isolated tubules with five concentrations of 1,9-dideoxyforskolin in the range 1.9×10^{-6} to 4.5×10^{-4} mol l^{-1}. 1,9-Dideoxyforskolin is believed to cause all the effects of forskolin save the central one of stimulating adenylate cyclase. None of these treatments with dideoxyforskolin caused any acceleration of fluid secretion by isolated tubules, providing evidence that forskolin acts by stimulation of adenylate cyclase.

In the presence of 10^{-5} mol l^{-1} forskolin, which by itself produces about 10% maximal stimulation, the sensitivity of the tubules to 5-HT is increased by some 30–50 times (Fig. 2); even 10^{-9} mol l^{-1} 5-HT causes a distinct acceleration of secretion.

A combination of 5×10^{-6} mol l^{-1} forskolin and 4×10^{-8} mol l^{-1} 5-HT, neither of

which by itself gave more than threshold stimulation, produced a near-maximal secretion rate, 858±47% (N=8) of the sum of the responses to each stimulant on its own (Fig. 3A), clear evidence of synergism of the two stimulants, each very likely to act by activation of adenylate cyclase.

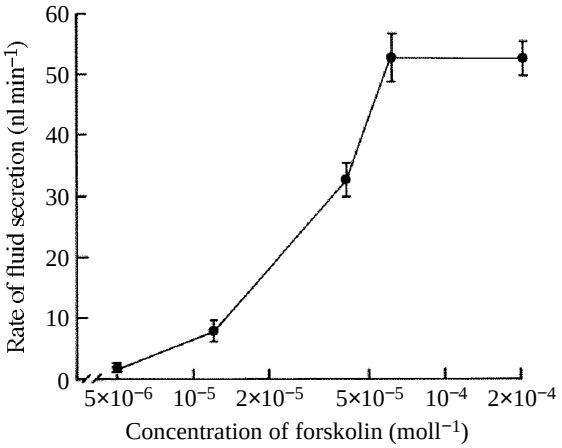

Fig. 1. Dose–response curve for stimulation by forskolin of fluid secretion by Malpighian tubules from fifth-instar *Rhodnius prolixus* in 125 μl drops of saline. Each point represents the mean rate over a period of 30min ± S.E. (N=12).

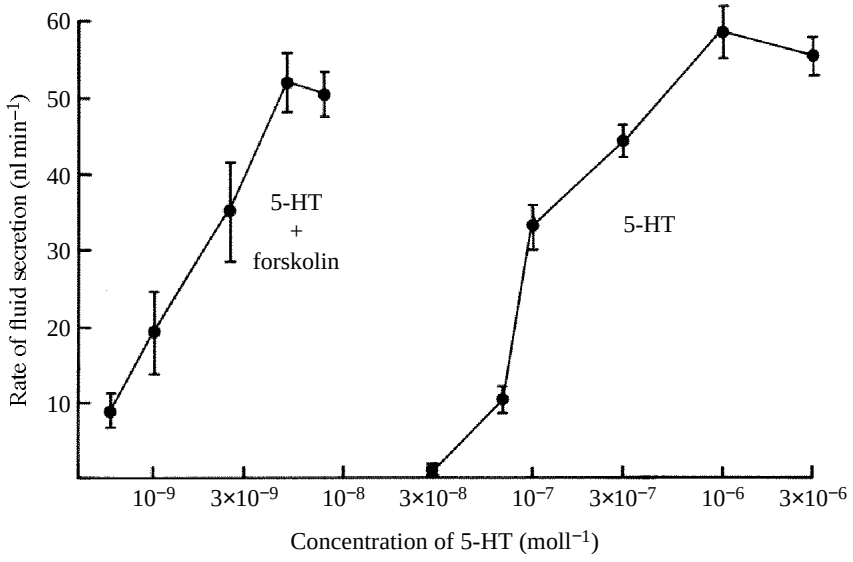

Fig. 2. Dose–response curves for stimulation of fluid secretion by Malpighian tubules from fifth-instar *Rhodnius* in 125 μl drops of saline, either by 5-HT alone (right-hand curve) or by 5-HT in the presence of 10^{-5} mol l^{-1} forskolin (left-hand curve). Each point represents the mean rate over a period of 30min ± S.E. (N=15). The rates seen at 3×10^{-7} mol l^{-1} are unusually low in this experiment; more typically, this concentration causes maximal secretion rates.

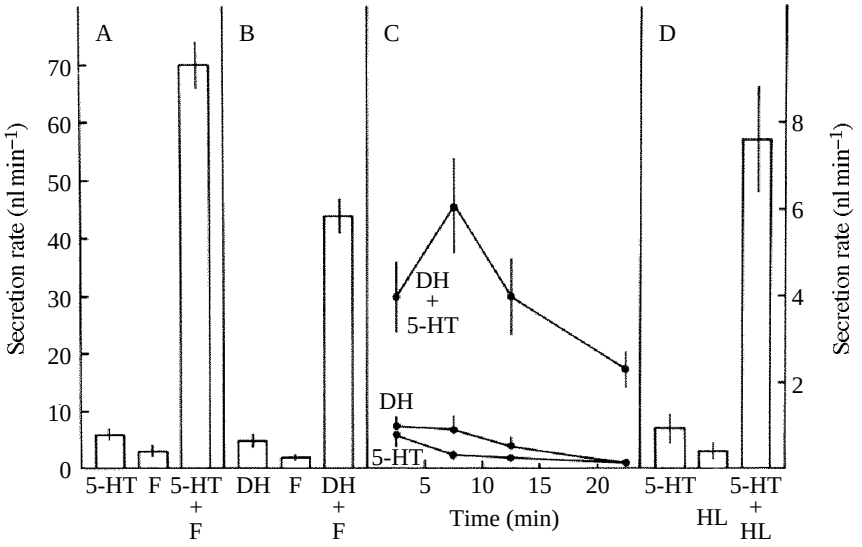

Fig. 3. Synergistic activation of *Rhodnius* Malpighian tubules by (A) 5×10^{-6} mol l^{-1} forskolin (F) and 4×10^{-8} mol l^{-1} 5-HT (*N*=8), (B) 5×10^{-6} mol l^{-1} forskolin (F) and extracts of the ganglionic mass (DH: 0.05 ganglionic masses per 100 μl), (C) 4×10^{-8} mol l^{-1} 5-HT and extracts of the ganglionic mass (DH: 0.05 ganglionic masses per 100 μl) and (D) 5×10^{-8} mol l^{-1} 5-HT and diluted haemolymph from recently fed fifth-instars (HL: 20%, diluted with standard saline). In A, B and C (left-hand axis), the tubules were from fifth-instar insects in 125 μl drops of saline, and in D the tubules were from third-instar insects in 30 μl drops, so the rates of fluid secretion were lower (right-hand axis). Each point represents the mean rate over a period of 30min (except for C) ± S.E.

Synergism between DH-rich ganglionic extracts and 5-HT or forskolin

The mesothoracic ganglionic mass and the proximal regions of the attached abdominal nerves of *Rhodnius* are a rich source of the peptide DH (Aston and White, 1974), but there is also present about 2pmol of 5-HT in each mass with its attached nerves (Lange *et al.* 1988). A threshold concentration of 0.05 ganglionic masses per 100 μl therefore contains 10^{-9} mol l^{-1} 5-HT, at which level 5-HT on its own is without effect (Fig. 2). A combination of this threshold concentration and 4×10^{-8} mol l^{-1} 5-HT caused secretion at a rate of 529±76% (*N*=8) of the sum of the separate responses to each stimulant (Fig. 3C), again clear evidence of synergism.

Similarly, combining 5×10^{-6} mol l^{-1} forskolin with 0.05 ganglionic masses per 100 μl caused secretion at a rate of 663±41% (*N*=8) of the sum of the separate responses (Fig. 3B).

Synergism between 5-HT and active haemolymph

Samples of haemolymph taken from insects 1–2h after feeding contain both peptide DH (Aston and White, 1974) and low levels of 5-HT (about 2×10^{-8} mol l^{-1}, Maddrell *et al.* 1991). 5×10^{-8} mol l^{-1} 5-HT combined with samples of haemolymph that had been diluted until they were almost inactive (in the case shown in Fig. 3D, this required a dilution to 20% with standard saline; the exact dilution used in any one experiment varied

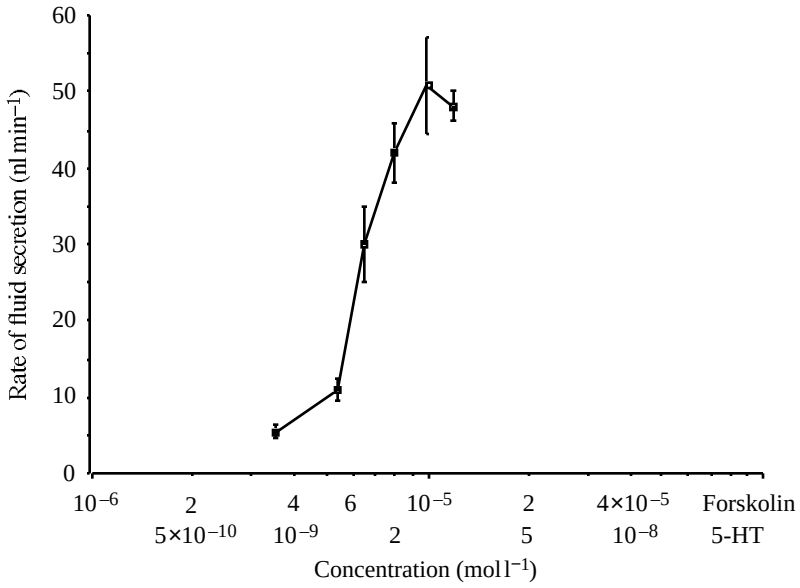

Fig. 4. Dose–response curve for stimulation of fluid secretion by Malpighian tubules from fifth-instar *Rhodnius* in 125 μl drops of saline containing a mixture of 5-HT and forskolin. Each point represents the mean rate over a period of 30min ± S.E. (*N*=6).

with the insects used and the time after feeding; the range of dilutions used varied from 15 to 40%), caused rates of secretion 581±91% (*N*=5) of the sum of the rates produced by each separately (Fig. 3D).

Steep dose–response curves for synergistic mixtures

A significant finding was that the dose–response curve for a mixture of forskolin and 5-HT was very much steeper (in terms of the ratio between increase in rate of fluid secretion and increase in concentration of stimulant) than for either stimulant on its own (Fig. 4). This raised the possibility that such a steep relationship is characteristic of a synergistic combination of stimulants. We found similarly steep dose–response curves for extracts of the ganglionic masses (Fig. 5) and, what is more important, for samples of active haemolymph (i.e. from recently fed insects), which is, of course, the natural stimulating medium of the tubules (Fig. 6). Each contains both peptide DH and 5-HT.

Fig. 6 emphasises the steeper relationship of stimulation by active haemolymph (in this case, haemolymph taken from insects fed 60min earlier) than by 5-HT. This experiment used 32 Malpighian tubules all taken on the same occasion from a single batch of eight third-instar insects (in each case, two of the four tubules from a particular insect were tested in diluted haemolymph and the other two in 5-HT-containing saline). All tubules were tested in 9 μl drops of saline-diluted haemolymph or of 5-HT-containing saline. The apparently higher sensitivity of the tubules to 5-HT in this experiment than in earlier ones (where tubules showed significant stimulation only above 4×10^{-8} to 5×10^{-8} mol l^{-1}

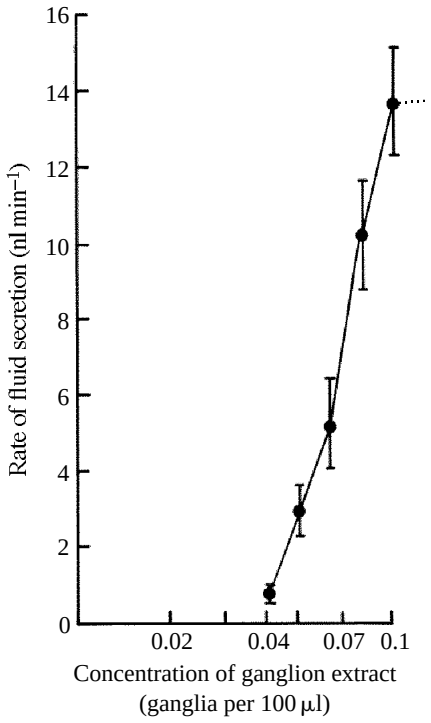

Fig. 5. Dose–response curve for stimulation of fluid secretion by Malpighian tubules from third-instar *Rhodnius* in 30 μl drops of saline containing different dilutions of an extract of mesothoracic ganglionic masses. Each point represents the mean rate over a period of 30min ± S.E. (*N*=4). Separate experiments showed that increasing the dose of ganglion extract above 0.1 ganglia per 100 μl did not further increase the rate of fluid secretion.

(Fig. 3A,C,D) and maximal stimulation at 2×10^{-7} mol l^{-1} 5-HT, data not shown) may be partly due to the use here of saline containing bicarbonate and phosphate (see Materials and methods). The necessity for replacing the saline stems from the fact that cells placed in salines that lack bicarbonate are not so well able to maintain their normal intracellular pH (Thomas, 1989). So our initial finding of steep dose–response curves from active haemolymph diluted with saline buffered only with Hepes might have been merely the result of progressive addition of a saline in which the tubule cells were increasingly less well able to function. The steep dose–response curve shown in Fig. 6 shows that this objection does not stand.

The data in Fig. 6 show that to accelerate fluid secretion by third-instar tubules from 1 to 9 nl min^{-1} with active haemolymph requires an increase in haemolymph concentration of around 50%, but needs a 260% increase in concentration of 5-HT to have the same effect. So the ratio between increase in rate of fluid secretion and increase in concentration of stimulant, i.e. the steepness of the dose–response curve, is about five times as high for active haemolymph as it is for 5-HT alone. The assertion that the dose–response curve for active haemolymph is steeper than that for 5-HT is an important one; similarly dramatic effects of haemolymph dilution, with halving of concentration

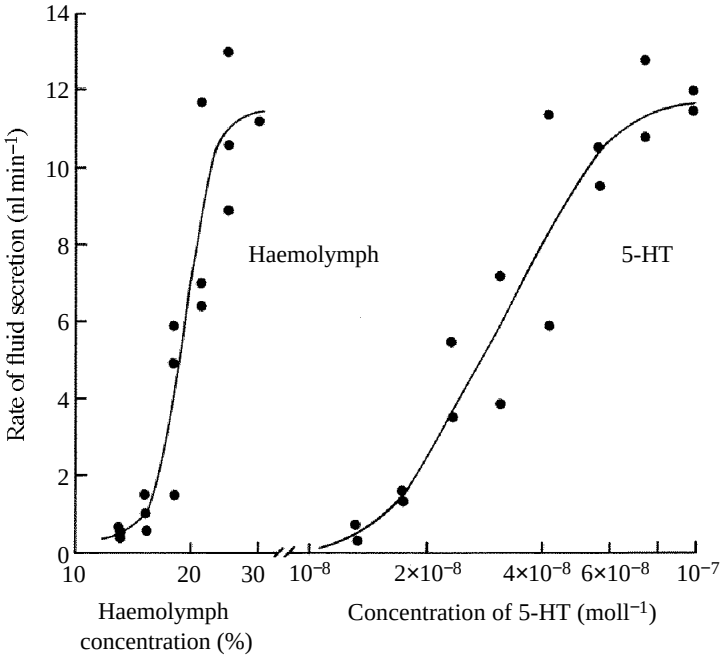

Fig. 6. Dose–response curves for stimulation of fluid secretion by Malpighian tubules from third-instar *Rhodnius* in 9 μl drops, either by 5-HT alone (right-hand curve) or by dilutions of haemolymph from fifth-instar insects fed 1h earlier (left-hand curve). Each point represents a determination of rate over the period between 20 and 30min after treatment.

causing almost complete loss of stimulation, have been observed in more than ten other experiments. The data shown in Fig. 6 are those of the most rigidly controlled experiment in which all possible differences between the tubules and the conditions in the two fluids were removed.

It is significant that we find that a 5-HT/forskolin mixture as well as extracts of the mesothoracic ganglionic mass and samples of haemolymph taken after feeding all have similarly steep dose–response curves (Figs 4, 5, 6). All contain 5-HT as one of their active constituents together with another stimulant; forskolin in the first case, the peptide DH in the other two cases. This argues that a steep dose–response curve is a characteristic feature of a combination of synergistic stimulants.

Activation of adenylate cyclase in broken membrane preparations from Malpighian tubules

Our results with forskolin, a stimulant of adenylate cyclase in many vertebrate systems (Seamon and Daly, 1982) and with peptide DH and 5-HT, both known to raise the level of intracellular cyclic AMP, raised the possibility that all three might act, at least in part, through an activation of adenylate cyclase. We measured the effects of ganglion extracts, 5-HT and forskolin on the activity of adenylate cyclase in broken membrane preparations from Malpighian tubules. The ganglion extracts were heated to

Table 1. *Effects of agonists on stimulation of adenylate cyclase from broken membrane preparations from* Rhodnius *Malpighian tubule cells*

	Agonist alone	Agonist + ketanserin ($5\,\mu mol\,l^{-1}$)	Agonist + GDPβS ($0.1 mmol\,l^{-1}$)
Forskolin	245±41	204±27	145±12
($30\,\mu mol\,l^{-1}$)	(3)	(3)	(3)
5-HT	435±18	91±15	9±3
($0.1\,\mu mol\,l^{-1}$)	(9)	(9)	(9)
Boiled ganglion extract	684±64	620±68	161±21
(1 mass per 100 μl)	(13)	(12)	(9)
Control	100±11	56±23	2±2
	(9)	(3)	(3)

Data are presented as mean± S.E.M. (*N*) as a percentage of the control value.

100 ˚C by holding the tubes containing the extracts in boiling water for 2–3min to destroy intrinsic adenylate cyclase activity. The stimulant activity of these boiled extracts on fluid secretion by isolated tubules was reduced to about 40% of that of untreated extracts. Aston and White (1974) found that such treatment largely destroyed the bioactivity of the diuretic peptide that they isolated. It seemed possible, therefore, that the residual activity that we detected was 5-HT, which is known to occur in the mesothoracic ganglionic mass. However, the residual fluid-stimulating activity was not affected by $10^{-6}\,mol\,l^{-1}$ ketanserin, which blocks 5-HT stimulation of *Rhodnius*' tubules (Maddrell *et al.* 1991). We presume that heating deactivates some of the different stimulant peptides that Aston and White (1974) found to be present in ganglion extracts, but not all of them. Forskolin, 5-HT and boiled ganglion extracts all stimulated adenylate cyclase (Table 1) in a dose-dependent manner (Fig. 7). The dose–response curve for the ganglion extracts is shifted to the right compared to that shown in Fig. 5 (by an amount which is not quantifiable as no determinations were made between 0.2 and 1.0 ganglia per 100 μl, where maximal stimulation is likely to have occurred), as expected from our finding that 60% of fluid-secretion-stimulating activity is lost on boiling. Otherwise the curves for activation of adenylate cyclase, given the difficulties of determination, are similar to those for stimulation of fluid secretion. Ketanserin greatly reduced the effect of 5-HT, but had no effect on forskolin or ganglion extract stimulation of the cyclase (Table 1). This confirms that activity in boiled ganglion extracts is not due to trace amounts of 5-HT. GDPβS, an inhibitor of guanine nucleotide binding proteins including G_S (Eckstein *et al.* 1979), reduced 5-HT stimulation by 98 %, but only decreased the stimulatory action of forskolin and ganglion extract by 41% and 76 % respectively. Finally, we measured adenylate cyclase activation by a mixture of boiled ganglion extract (0.1 ganglionic masses per 100 μl) and 5-HT ($10^{-8}\,mol\,l^{-1}$). The extract by itself slightly activated the cyclase (an increase of 49±27%, N=3) as did $10^{-8}\,mol\,l^{-1}$ 5-HT (an increase of 38±16%, N=3), but a mixture of the two caused marked cyclase activation, to a level 515±66% (N=3) of that of non-stimulated controls, providing evidence of synergism between the two stimulants.

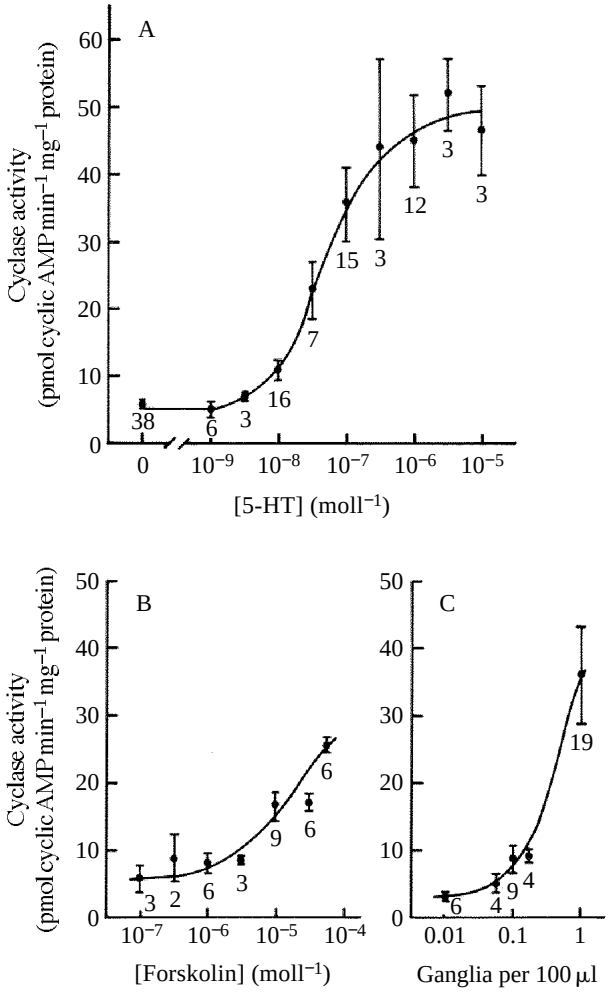

Fig. 7. Dose–response curves for stimulation of adenylate cyclase from broken membrane preparations from fifth-instar Malpighian tubule cells by (A) 5-HT, (B) forskolin and (C) boiled extracts of ganglionic masses (DH). Each point represents the mean activity ± S.E. (the number of determinations is shown beside each point).

Experiments with 5,7-dihydroxytryptamine

5,7-Dihydroxytryptamine (5,7-DHT) is an accepted tool for 'chemical degeneration' of serotonergic (5-HT) axons in the central nervous system of vertebrates (Baumgarten *et al.* 1982). In *Rhodnius*, injection of 5,7-DHT causes a decrease in the staining intensity of serotonin-like endings on the abdominal nerves and a severe decrease in the 5-HT content of the nerves, demonstrating that 5,7-DHT is an effective neurotoxin of peripheral 5-HT stores in this insect (Cook and Orchard, 1990). We injected fifth-instar insects through a mesothoracic leg with 1–1.5 µl of 10^{-2} mol l^{-1} or 10^{-3} mol l^{-1} 5,7-DHT in 0.001% ascorbic acid and fed the insects 24h later. Just as Cook and Orchard (1990) found, the treated insects fed abnormally. They started to feed but stopped after they had taken about

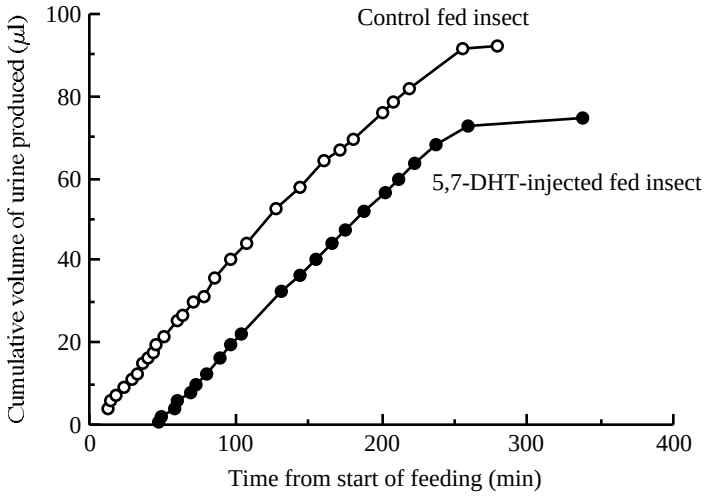

Fig. 8. Diuresis in a control fed fifth-instar *Rhodnius* (open circles) and in one previously injected with 5,7-DHT (filled circles).

50–100mg of blood. However, after a further minute or two, they usually began feeding again and went on to take meals nearly as large as uninjected controls. The abdominal cuticle then showed abnormal plasticisation: regions of it, usually the more anterior parts, did not become as transparent as usual, reminiscent of the effects of cutting the nerves to parts of the abdominal wall (Maddrell, 1966). Particularly with insects injected 24 h earlier with 10^{-2} mol l^{-1} 5,7-DHT, the diuresis that normally begins as soon as 3min after feeding begins (Maddrell, 1964) was delayed or, in some cases, did not occur at all. Of 23 such injected insects, diuresis was entirely prevented in five and delayed by a period of between 10 and 50min in the remainder (Fig. 8). In those insects in which there was a delay, the eventual diuresis was apparently normal in both rate and extent. In 10 fed control insects, injected 24h earlier with 1.5 μl of 0.001% ascorbic acid alone, diuresis was normal. To confirm that potential 5-HT release from the neurohaemal areas in 5,7-DHT-treated insects had been reduced in our experiments, we treated groups of four mesothoracic ganglionic masses with attached abdominal nerves (on which lie the neurohaemal areas for 5-HT release, Flanagan, 1984) from 5,7-DHT-treated fifth-instar *Rhodnius* with K^+-rich saline (70mmol l^{-1} K^+; 40 μl drops; 20min). In two such experiments, assay of the drops for 5-HT content with isolated salivary glands of *Calliphora erythrocephala*, known to be very sensitive to 5-HT (Berridge and Patel, 1968; Berridge, 1970), showed that the treatment caused only 25% as much 5-HT release from 5,7-DHT-treated ganglia as from groups of ganglia from untreated control insects. Cook and Orchard (1990) showed that 24h after injection of 5,7-DHT, the 5-HT concentration in the abdominal nerves was similarly reduced compared with controls.

Discussion

The major finding of this paper could be said to be that shown in Fig. 6; that is, that the combination of more than one natural stimulant (of fluid secretion by the Malpighian

tubules) in the haemolymph of fed insects is characterised by a very steep dose–response curve. Similar steep dose–response curves can be produced by fluids containing only the two stimulants 5-HT and forskolin. Samples of active haemolymph from recently fed insects contain both 5-HT and the peptide diuretic hormone DH. It is tempting to argue, therefore, that it is the synergistic action of these two hormones that explains the steepness of the dose–response curve of active haemolymph. Although this is the simplest explanation of the results, the possibility cannot be ruled out that the situation is more complicated. Conceivably, other active compounds that might be present in the haemolymph could be involved. Nonetheless, we find steep dose–response curves for stimulation of tubule fluid transport only in cases where there is *more* than one stimulant present, but such a steep curve can be produced by a fluid with just two stimulants; however, where there is only one stimulant, be it cyclic AMP, forskolin or 5-HT, the dose–response curve is always markedly less steep. We can reasonably conclude that the natural stimulating environment (the haemolymph in recently fed insects) must contain more than one stimulant. The most economical hypothesis is that 5-HT and the peptide diuretic hormone, both now known to be diuretic hormones (Maddrell *et al.* 1991), act synergistically to control fluid secretion by the Malpighian tubules in the period after a blood meal. The involvement of three or more substances, with the difficulties of regulating such an array, seems a less attractive proposal. To establish whether we need suppose that stimulants other than 5-HT and the peptide DH are involved, we must wait for experiments with mixtures of purified peptide DH and 5-HT. The difficulties of isolating and purifying the peptide hormone in the form in which it is circulated make this a daunting prospect. Our experiments with 5,7-DHT-treated insects show that interference with 5-HT release causes a much slower diuretic response after feeding, suggesting that 5-HT, at least, has an important rôle in normal rapid diuresis.

The interaction of 5-HT and the peptide hormone may not be limited to synergism in causing very fast fluid secretion by the Malpighian tubules. Flanagan and Berlind (1984) showed that 5-HT caused the release of tritiated 5-HT previously taken up into the neurohaemal sites on the abdominal nerves in *Rhodnius*. They went on to raise the intriguing possibility that 5-HT released into the haemolymph after feeding might not only accelerate its own release, but also act to stimulate release of the peptide hormone DH. This would hasten the attainment of levels of each at which they would stimulate the activity of the Malpighian tubules, making the diuretic response still faster.

Our finding of a synergistic cooperation of different stimulant substances, both in greatly accelerating fluid secretion by the Malpighian tubules and in stimulating adenylate cyclase from the tubules, is the first evidence of such a novel regulatory system in insects. What are the conceivable advantages of such an apparently complex control system involving more than one hormone? In the discussion that follows, we treat the idea that it is two hormones that are involved, but the arguments equally apply to the synergistic cooperation of more than two. One obvious advantage is that if *low* concentrations of two hormones can produce the same effects as a *high* concentration of just one of them, then there is a saving in the total amount of hormone that has to be released into circulation. Perhaps it is the case that if the tubules respond to two signals rather than just one, then this may improve the signal to noise ratio and so improve the

reliability of the response. It also follows that the speed of response to feeding can be greater because it takes less time to release small amounts of two hormones than a more substantial amount of just one, particularly if one of them has stimulatory feedback effects on release, as for example in 5-HT release in *Rhodnius* (Flanagan and Berlind, 1984). This is likely to be of particular usefulness in insects, because of their relatively sluggish circulation.

We have argued that one of the hallmarks of synergistic action of two stimulants is the steepness of the dose–response curve for a mixture of the two. Such a steep relationship will improve control, because a small change in concentration of the two will produce a large effect, giving an almost on/off switch. The oxygen–haemoglobin binding curve is similarly steep, as are those describing the effects of allosteric activators on enzyme activity; in both cases precision of regulation and action are also important. Steep dose–response curves are also found for fast neurotransmitters that directly activate ion channels (Hardie, 1989). Their action involves cooperativity, but also low affinity, quite unsuitable for hormones present in circulation at low concentration. Synergism of two hormones allows this limitation to be overcome and still provides the benefits of rapid action with a steep dose–response curve.

Rapid initiation of diuresis in *Rhodnius* may, therefore, depend on the simultaneous release of, at least, 5-HT and the peptide DH. A further benefit is that relatively minor changes in the concentrations of the two substantially alter the rate of diuresis and this may be useful not only in the initiation of diuresis but also in its rapid termination after a sufficient volume of urine has been eliminated.

An important advantage to *Rhodnius* of using 5-HT in conjunction with the peptide DH to control the rate of fluid secretion by its Malpighian tubules is that the concentration of 5-HT in circulation need never be very high. The significance of this is that 5-HT is a very active substance which, at a concentration high enough to stimulate maximal secretion by the Malpighian tubules, would have pronounced effects on a variety of other physiological systems in *Rhodnius*. For example, 5-HT stimulates fluid absorption by the midgut (Farmer *et al.* 1981), KCl uptake in the lower Malpighian tubule (Maddrell and Phillips, 1975), the heart beat and rate, the plasticity of the abdominal cuticular wall (Reynolds, 1974) and the rate of contraction of muscles of the alimentary canal; there may well be yet other 5-HT-sensitive systems. So, release of a relatively high concentration of 5-HT would activate many systems in the insect in an inappropriate and possibly damaging way. With a synergistic coupling of 5-HT and DH, only a modest level of 5-HT is needed. One can speculate that the rôle of 5-HT is to sensitise particular systems, such as the Malpighian tubules, so that they can respond quickly to low levels of more specific hormones. On this view, 5-HT would be an arousal hormone. This fits well with the earlier suggestion made by Barrett and Orchard (1990) that the rôle of 5-HT might be to act as an overall coordinator of feeding activity. Once diuresis is under way, the level of 5-HT is reduced within minutes to around 1×10^{-8}–2×10^{-8} mol l^{-1} from the maximal level of about 5×10^{-8} mol l^{-1} reached shortly after feeding begins (Lange *et al.* 1989; Maddrell *et al.* 1991). The maintained level of 5-HT is, by itself, scarcely sufficient to cause any elevation in the rate of fluid secretion by the Malpighian tubules.

The discovery, in *Rhodnius*, of synergism between (at least) two hormones raises the

question of whether such cooperative action might be found in other insects. One such instance may occur in the salivary response of adult blowflies. When they settle to feed, they secrete the saliva they need at the time, rather than release it from a reservoir. To do this they activate the salivary glands with 5-HT (Trimmer, 1985). Given our finding that faster activation can be achieved with two synergistic hormones, we are encouraged to speculate that there may be release of a peptide hormone simultaneously with 5-HT in feeding adult blowflies.

More generally, it has been found that 'arousal' in a whole range of insects involves the release of octopamine and/or a peptide hormone (Corbet, 1991), raising the possibility that both are released simultaneously to achieve 'arousal'. Perhaps the amine and peptide cooperate synergistically in these cases just as 5-HT and DH do in *Rhodnius*. Interestingly, the 5-HT-containing cells of *Rhodnius* are the so-called DUM cells (*d*orsal *u*npaired *m*edial cells) (Orchard *et al.* 1989). All other known DUM cells in insects contain octopamine and not 5-HT. *Rhodnius* seems to be unique in having 5-HT-containing DUM cells; conceivably that may be why diuresis is controlled in this insect by 5-HT and a peptide, rather than by octopamine and a peptide. It may be relevant that 5-HT is available to *Rhodnius* from the platelets in its diet of vertebrate blood (Da Prada and Picotti, 1979). It will be of great interest to find out whether mixtures of octopamine and peptide hormone act synergistically to control physiological systems in those insects that have octopamine-containing DUM cells.

Finally, it is worth considering what implications control by 5-HT and DH acting jointly might have for the other components of the diuretic response in *Rhodnius*. At feeding, blood is first taken into the expanded part of the midgut, the functional crop (Wigglesworth, 1972). From there, a NaCl-rich, iso-osmotic fluid is absorbed into the haemolymph (Farmer *et al.* 1981). The upper fluid-secreting lengths of the Malpighian tubules take up fluid from the haemolymph and secrete it into their lumina. This fluid, containing both NaCl and KCl, passes through the lower lengths of the tubules, where KCl is very rapidly reabsorbed (Maddrell and Phillips, 1975). Both fluid absorption by the midgut and KCl reabsorption by the lower Malpighian tubules can be greatly accelerated by 5-HT (Farmer *et al.* 1981; Maddrell and Phillips, 1975). An obvious possibility is that these other epithelia may be controlled synergistically by 5-HT acting jointly with the peptide DH.

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