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Effects of corpora cardiaca extract, furosemide and ion substitution on sodium and chloride flux in perfused Malpighian tubules of *Locusta*

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Abstract. Treatment with the co-transport inhibitor, furosemide decreased $^{36}Cl^-$ flux across perfused Malpighian tubules of *Locusta*. However, exclusion of $^{36}Cl^-$ from the bathing medium had no effect on $^{22}Na^+$ flux whereas substitution of bathing medium Na^+ by K^+ increased $^{36}Cl^-$ flux. Diuretic extract of corpora cardiaca increased $^{22}Na^+$ (by 106%) and $^{36}Cl^-$ (by 335%) fluxes differentially.
Key words. *Locusta*; Malpighian tubules; ion flux; furosemide; corpora cardiaca.

Fluid secretion by Malpighian tubules of many insect species is dependent on active ion transport[1–3], with K^+ secretion acting as the 'prime mover'. It is generally accepted that an electrogenic cation pump is located at the apical cell membrane[4–7] and is responsible for the transport of K^+ and/or Na^+ from the cytosol into the tubule lumen. The movement of Cl^- from cytosol to tubule lumen is thought to be passive, resulting from the large, favourable electrical gradient. Measurements of potential differences across the basal membranes (V_b) of the tubule cells of *Locusta*[7], combined with ion substitution, indicate that the basal cell membrane is permeable to K^+, but relatively impermeable to Na^+ and Cl^-. Potassium ions must enter the cell across the basal membrane to provide the basis of the K^+-rich 'urine' which is secreted by Malpighian tubules of *Locusta*[3]. Estimates of the potassium equilibrium potential (E_K) yield values somewhat more negative than the measured V_b suggesting that K^+ entry across the basal plasma membrane is unlikely to be accounted for by passive permeability[7]. Furthermore, whilst the electrochemical gradient favours Na^+ entry into the cell, it opposes Cl^- influx. Some mechanism must exist, therefore, to transport Cl^- and K^+ into the cell. K^+ will enter, in part, by exchange for intracellular Na^+ by means of the basolateral Na^+/K^+ ATPase pump; an enzyme known to be present in microsomal preparations of *Locusta*[8,9]. In *Rhodnius*[6] and *Aedes*[10] it is suggested that Cl^- entry is dependent on an electroneutral co-transport with Na^+ and K^+, driven by the favourable inward gradient for Na^+; the suggested stoichiometry[6] being $Na^+:K^+:2\,Cl^-$. It is possible that a similar mechanism exists in *Locusta*. However, microelectrode studies involving ion substitution suggest that in *Locusta* the role of Na^+ in such a mechanism is less certain and the evidence points to a K^+-dependent Cl^- entry process[7]. Such a K^+-dependent Cl^- entry process has been reported in locust rectum[11] and tentatively suggested in Malpighian tubules of *Locusta*[12]. However, a furosemide-sensitive K-Cl cotransport mechanism such as that reported in mammalian erythrocytes[13] is another possibility.

Morgan and Mordue[12] have suggested that one of the actions of diuretic hormone is to stimulate coupled anion-cation transport across the basal membrane of *Locusta* tubule cells. Similarly, the agonist 5-hydroxytryptamine (5-HT) is thought to stimulate co-transport across the basal cell membrane in *Rhodnius*[6]. The present study has been carried out to determine the extent to which Na^+ and Cl^- flux across the Malpighian tubules of *Locusta* are linked and how these ionic fluxes are affected by corpora cardiaca extracts with diuretic activity.

Materials and methods

The insects used were sexually mature locusts, *Locusta migratoria* L., reared under standard conditions described previously[3].

Animals were killed by decapitation and the Malpighian tubules dissected free of the alimentary tract in standard Ringer solution (see table 1). Individual tubules were transferred to a liquid paraffin bath with a Sylgard base and secured under liquid paraffin at both ends; one end being wrapped around a vertical glass pin and the other end being held by a pair of adjustable mounted forceps. Luminal perfusion was carried out essentially as described by O'Donnell and Maddrell[14]. A fine glass pipette, which served as a cannula, was then inserted into the lumen of the tubule close to the retaining forceps and directed along its length. The tubule was partially severred at its other end, near the glass pin. The cannula was filled with a perfusion medium of the following composition (in mM): NaCl 9.0; KCl 99.6; $MgCl_2$ 8.5; $CaCl_2$ 2.0; NaH_2PO_4 4.0; $NaHCO_3$ 4.0, glucose 40; NaOH 11; HEPES 25.0 (pH 7.2) and connected to a motor-driven microsyringe. Positive

Table 1. Composition of salines (concentration in mM/l)

Salt	Standard saline	High $[K^+]$, Na^+-free	Cl^--free
NaCl	100.0	-	-
KCl	8.6	108.6	-
$CaCl_2$	2.0	2.0	-
$MgCl_2$	8.5	8.5	-
NaH_2PO_4	4.0	-	4.0
$NaHCO_3$	4.0	-	4.0
NaOH	11.0	-	11.0
Glucose	34.0	34.0	34.0
HEPES	25.0	25.0	25.0
KH_2PO_4	-	4.0	-
$KHCO_3$	-	4.0	-
KOH	-	11.0	-
Na gluconate	-	-	100.0
K gluconate	-	-	8.6
Ca gluconate	-	-	2.0
Mg gluconate	-	-	8.5

All solutions were adjusted to pH 7.2.

pressure, applied to the syringe plunger, ejected any luminal contents at the site of partial severance and reduced the possibility of tubule blockage. Care was taken to ensure that there was no leakage along the length of the tubule before proceeding. Once a suitable tubule was established, a 100-µl droplet of standard Ringer solution was placed in a small well such that it surrounded a portion of the tubule. The surface area of tubule exposed to this solution was calculated by measuring the length (L) and diameter (D) of this Ringer-bathed region and treating it as a cylinder (i.e. surface area bathed $= \pi DL$). The tubule lumen was then perfused at a constant rate of ca 50 nl/min using the motor-driven syringe. Net flux of Na^+ and Cl^- from the well (haemolymph side) to the tubule lumen was determined using ^{22}Na and ^{36}Cl, respectively. The perfusion medium emerging as a droplet at the site of partial severance was collected after 10 min, placed in 10 ml of 'Liquiscint' scintillation fluid and the radioactivity measured in a Canberra Packard Tri-Carb 300 scintillation counter; the level of radioactivity being proportional to the total amount of Na^+ or Cl^- flux across the bathed tubule surface. Flux rates were calculated as mequiv. Na^+ or Cl^- per cm^2 tubule $\cdot h^{-1}$ and are presented as mean $\pm$SEM, unless otherwise stated.

All experiments were carried out at 30 ± 0.1 °C. The bathing medium was standard Ringer solution, unless otherwise stated. For each tubule, the initial flux rate was determined. The external bathing droplet was then changed for a fresh medium (table 1) which had either the same (control) or a different composition (experimental) and included the appropriate radioisotope. The flux rate was then redetermined. In this way, the effect of individual variation between different tubules could be minimized. In studies involving furosemide it was necessary to dissolve it in ethanol before adding it to the appropriate saline. Consequently, the same concentration of ethanol ($<0.1\%$) was included in the controls for these experiments.

All inorganic salts were AnalaR grade or the best commercially available and solutions were made up in glass-distilled, deionized water. ^{22}Na and ^{36}Cl were both obtained as sodium chloride in aqueous solution (Amersham International Ltd). Liquiscint was supplied by National Diagnostics, New Jersey, USA..

Corpora cardiaca extracts with established diuretic activity were prepared immediately prior to use, as described previously[15]. Such extracts were applied to the tubules in appropriate saline solutions at a concentration of 1 gland pair per cm^3.

Results

Mean net flux rates, from bathing medium to lumen, for Na^+ ($^{hl}Na^+$) and Cl^- ($^{hl}Cl^-$) were 0.64 ± 0.07 mequiv. per $cm^2 \cdot h^{-1}$ ($n = 26$ independent determinations) and 1.02 ± 0.19 mequiv. per $cm^2 \cdot h^{-1}$ ($n = 27$ independent determinations), respectively. No significant differences were observed in controls between flux rates observed over the initial and final periods of measurement, for either ion. In contrast, inclusion of crude corpora cardiaca extract in the bathing medium (standard Ringer solution) significantly increased $^{hl}Na^+$ flux (see table 2) from 0.67 ± 0.11 mequiv. per $cm^2 \cdot h^{-1}$ to 1.25 ± 0.26 mequiv. per $cm^2 \cdot h^{-1}$, representing an increase of approximately 106%. Similarly, $^{hl}Cl^-$ flux increased from 0.79 ± 0.28 mequiv. per $cm^2 \cdot h^{-1}$ to 3.09 ± 0.87 mequiv. per $cm^2 \cdot h^{-1}$ (table 2), representing an increase of approximately 335%.

On changing the bathing medium from standard to Cl^--free saline, there was no significant change in $^{hl}Na^+$ flux (table 2) suggesting that movements of Na^+ and Cl^- across the tubule wall are not linked. Nevertheless, changing the bathing medium from standard Ringer solution to a high $[K^+]$, Na^+-free saline (table 2) significantly increased $^{hl}Cl^-$ flux ($p < 0.01$) by a mean value of 0.45 ± 0.1 mequiv. per $cm^2 \cdot h^{-1}$ from 0.50 ± 0.04 mequiv. per $cm^2 \cdot h^{-1}$ to 0.95 ± 0.09 mequiv. per $cm^2 \cdot h^{-1}$, representing a mean increase of approximately 95%.

Inclusion of 1 mM furosemide in standard Ringer solution bathing the tubule effected a significant decrease (table 2) in $^{hl}Cl^-$ flux by a mean value of 0.24 ± 0.25 from an initial rate of 0.44 ± 0.09 mequiv. per $cm^2 \cdot h^{-1}$ to 0.20 ± 0.04 mequiv. per $cm^2 \cdot h^{-1}$; a decrease of approximately 48%.

Discussion

In the present study, net Cl^- flux from the bathing medium to lumen was greatly reduced following treatment with the 'loop' diuretic furosemide. Furosemide is thought to inhibit Na/K/Cl or Na/Cl cotransport in various epithelia[11,16–19]. It would appear, therefore, that

Table 2. Effects of ion substitution, corpora cardiaca extract and furosemide on Na^+ and Cl^- flux across Malpighian tubules of *Locusta*.

Treatment	Ion	Flux rate (mequiv. per $cm^2 \cdot h^{-1}$)		n	p
		Initial	Final		
CC extract in standard saline	Na^+	0.67 ± 0.11	1.25 ± 0.26	12	<0.002
	Cl^-	0.79 ± 0.28	3.09 ± 0.87	6	<0.05
Cl^--free saline	Na^+	0.73 ± 0.10	0.77 ± 0.11	6	n.s.
High $[K^+]$, Na^+-free saline	Cl^-	0.50 ± 0.04	0.95 ± 0.09	6	<0.01
1 mM-furosemide in standard saline	Cl^-	0.44 ± 0.09	0.20 ± 0.04	7	<0.02

p were obtained by comparing initial and final flux rates by a t-test for paired data. n represents the number of independent determinations. In all cases, initial flux rates were determined in standard saline prior to the treatment indicated.

one or other of these cotransport processes is operating in Malpighian tubules of *Locusta*. However, the fact that the net flux of Na^+, from bathing medium to lumen, was unaffected by removal of external Cl^- raises questions concerning the role of Na^+ in Cl^- movement across the epithelium. Similarly, Cl^- flux was not significantly reduced in the absence of Na^+. Both these observations suggest that movements of Na^+ are not strongly linked to those of Cl^-. Baldrick et al.[7] suggested that some Cl^- entry into the cell, across the basal plasma membrane, may be possible through cotransport with K^+ under Na^+-free conditions. A similar mechanism has also been proposed for *Rhodnius prolixus*[6] where, in the absence of either cation, the $Na^+:K^+:2\,Cl^-$ cotransporter may accept, though less readily, other stoichiometries such as $2\,Na^+:2\,Cl^-$ or $2\,K^+:2\,Cl^-$. In Malpighian tubules of *Locusta migratoria*, Cl^- flux from bathing medium to lumen was significantly increased under high $[K^+]$, Na^+-free conditions suggesting that the above Cl^- entry mechanism can operate independently of Na^+, but may be dependent on external K^+ concentration. A K^+-dependent Cl^- pump has been reported in locust rectum[11] and tentatively suggested in Malpighian tubules of *Locusta*[12]. Cl^- entry via such a K^+-dependent Cl^- pump is not inconsistent with the results of this investigation. Indeed, elevating external $[K^+]$ would be expected to lead to increased activity of such a pump and to increase the transport of Cl^- into the cell. This would also be consistent with data from microelectrode studies[7]. Alternatively, it could be that Cl^- entry into the Malpighian tubule cells of *Locusta* is by means of a distinct furosemide-sensitive K/Cl cotransport mechanism similar to that reported in mammalian erythrocytes[13,20], *Necturus* gallbladder[21] and rabbit renal cortical basolateral membrane vesicles[22] or some aspect of the Na/K/Cl cotransporter. Some workers believe that the various transport systems which are inhibitable by furosemide, for example $K^+:Cl^-$ and $Na^+:Cl^-$ cotransport, may represent a partial aspect of the $Na^+:K^+:2\,Cl^-$ cotransporter rather than representing a distinct system[23]. In control and Cl^--free salines, $[Na^+]$ levels are high (119 mM) compared to $[K^+]$ (8.6 mM), conditions which, according to O'Donnell and Maddrell[6], would favour $Na^+:K^+:2\,Cl^-$ or $Na^+:Cl^-$ cotransport. This does not appear to be the case in *Locusta*.

The effect of corpora cardiaca extract on the tubules was to significantly increase both the net $^{hl}Na^+$ and $^{hl}Cl^-$ fluxes by approximately 106% and 335%, respectively. These results are clearly incompatible with stimulation of a cotransport of these two ions; again, if Na^+ and Cl^- movements were linked, one would expect stimulation by corpora cardiaca extract to result in the same percentage increase in net flux for both of these ions. Thus the evidence would seem to support the existence of a $K^+:Cl^-$ cotransport mechanism in the basal membrane of the Malpighian tubule cells of *Locusta*. However, for tubules bathed in control saline the question arises as to how such a $K^+:Cl^-$ cotransporter could operate due to the electrochemical gradients for both K^+ and Cl^- favouring their exit from the cell across the basal plasma membrane[7]; i.e. there is no driving force from the transport of Cl^- into the cell by such a mechanism. Thus, whilst a $K^+:Cl^-$ cotransporter might operate when $[K^+]_0$ is elevated, it is difficult to see such a mechanism working at ionic levels normally found in haemolymph. It would appear, therefore, that the effect of furosemide in control saline, on Cl^- flux across the tubules of *Locusta*, is best explained by its relatively non-specific action. Furosemide is reported to inhibit other Cl^- transporting processes[19], $(Na^+ + K^+)$-ATPase[24], to reduce passive Cl^- permeability[25] and to inhibit carbonic anhydrase. In conclusion, the evidence presented above argues against the linkage of Na^+ and Cl^- by cotransport across the basal cell membrane of Malpighian tubule cells of *Locusta* as the main mechanism for ion entry.

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