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ATPase ACTIVITY IN THE MIDGUT OF THE MOSQUITO, *ANOPHELES STEPHENSI*: BIOCHEMICAL CHARACTERISATION OF OUABAIN-SENSITIVE AND OUABAIN-INSENSITIVE ACTIVITIES

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Summary

Na^+/K^+-ATPase activity was demonstrated in the midgut of *Anopheles stephensi.* More than 80% of the total ATPase activity was sensitive to inhibition by ouabain with an IC_{50} of $4.5\times10^{-7}\pm0.3\times10^{-7}$ mol l^{-1} and with maximal inhibition occurring at 10^{-4} mol l^{-1}. This ouabain-sensitive Na^+/K^+-ATPase was maximally activated at a Mg^{2+}:ATP ratio of 1:1.3, with a K_m of 0.3 mmol l^{-1} and a V_{max} of 2.4 μmol P_i mg^{-1} protein min^{-1} for ATP. Maximal activation was reached at 15 mmol l^{-1} K^+ with a K_m of 0.72 mmol l^{-1}. Activation with Na^+ showed an increase up to 120 mmol l^{-1} with a K_m of 6.47 mmol l^{-1}, and the optimal K^+:Na^+ ratio was 1:5.5. The ouabain-sensitive enzyme was inhibited by Ca^{2+} with an IC_{50} of 1.11±0.07 mmol l^{-1}. The pH optima were 7.2 for the ouabain-sensitive enzyme and 8.9 for the ouabain-insensitive fraction. The minor ouabain-insensitive fraction was unaffected by Na^+, K^+ or Ca^{2+}, but was dependent to some extent on Mg^{2+}. The demonstration of a ouabain-sensitive Na^+/K^+-ATPase being a major ATPase in the mosquito midgut is consistent with the hypothesis that this region is actively involved in post-feeding ion and water regulation.

Introduction

In common with other blood-feeding insects, the malaria vector, *Anopheles stephensi*, undergoes a period of rapid ion regulation and fluid transport during and subsequent to ingestion of a blood meal. This pre-diuresis, visible in the form of excreted droplets, is clearly a major physiological event. Although much is now known about protein digestion in *A. stephensi* (Billingsley, 1990*a,b*; Feldmann *et al.* 1990), the physiological mechanisms involved in ion and water regulation have not been identified or characterised. The Na^+/K^+-ATPase is the plasma-membrane-associated enzyme which catalyses ubiquitous, ATP-driven Na^+/K^+ transport. This enzyme is crucial to ion and water regulation in the vertebrate kidney and is an apparently universal feature of secretory epithelia as well as of excitable tissues. In ion-transporting epithelia, Na^+/K^+-

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ATPase is restricted to the basolateral plasma membrane, thus allowing directed fluid transport to occur.

Na^+/K^+-ATPase is a P-type ATPase, so called because ATP hydrolysis proceeds *via* a phosphorylated enzyme intermediate. An essential feature of Na^+/K^+-ATPase action is that cation transport is consecutive, Na^+ transport preceding K^+ transport (Karlish, 1989). The enzyme consists of an $\alpha\beta$ heterodimer which comprises the minimal functional unit able to hydrolyse ATP and undergo enzyme transitions (Jorgensen and Anderson, 1988). The 110×10^3 M_r α-subunit contains the catalytic binding sites and requires ATP, Mg^{2+} and Na^+ on its cytoplasmic side and K^+ on its extracellular side for activation (Rossier *et al.* 1987; Skou, 1988). Although Na^+/K^+-ATPases in different tissues are not identical, there is a 95% homology between α-subunits and a 60% homology between β-subunits, and the transmembrane arrangements appear to be similar across species (Kawakami *et al.* 1985; Ovchinnikov *et al.* 1986; Ovchinnikov, 1987; Shull *et al.* 1985). Although the direct matching of isoforms to tissue types is still in its infancy, several isoforms of the enzyme with varying ouabain sensitivities have now been recognised in different species, in different tissues of individuals and within a single tissue over a developmental period (Lucchesi and Sweadner, 1991; Maixent *et al.* 1991; Sweadner, 1989).

Na^+/K^+-ATPase activity has been characterised in some insect tissues (Anstee and Bowler, 1984) and been shown to be essential to excretory functions (Anstee and Bell, 1975; Farmer *et al.* 1981; Peacock, 1982). In the haematophagous dipteran *Glossina morsitans,* a ouabain-sensitive Na^+/K^+-ATPase has been localised in the anterior midgut region, with high activity relative to other gut regions (Peacock, 1981). Physiological and ultrastructural studies in *G. morsitans* have established this region as the site of rapid water absorption after ingestion of a blood meal (Brown, 1980; Ramsay, 1950; Treherne, 1962, 1967). Functional characteristics of Na^+/K^+-ATPase activity in the midgut of mosquitoes have never previously been investigated. Ultrastructural studies have, however, suggested that the posterior midgut epithelium of mosquitoes is a site of ion and water transport (Billingsley, 1990*b*). In view of the importance of *A. stephensi* as a disease vector and the physiological significance of ion and water regulation in this insect, the midgut environment is a potential target site for both vector and parasite control (Ramasamy *et al.* 1988; Sinden, 1984). The present work has been initiated with the aim of biochemically characterising Na^+/K^+-ATPase activities in the mosquito midgut as part of an overall elucidation of ionic mechanisms, water transport processes and functional cell biology in the digestive system of haematophagous insects.

Materials and methods

Mosquitoes

Anopheles stephensi SDA 500 strain, originally obtained from the University of Nijmegen (The Netherlands), were reared in a 12h:12h light:dark cycle at 75–80 % relative humidity and 27˚C. Eggs were hatched in distilled water containing $0.1\,\mathrm{g\,l^{-1}}$ mixed sea salts. Larvae were fed *ad libitum* on Liquifry (first instar), Tetra-Min (second instar) and Omega Pond Fish food pellets (size 5) (third and fourth instars). Pupae were separated and adults allowed to emerge directly into a cage. Adults were maintained *ad*

libitum on 8% fructose containing 0.1% PABA (para-aminobenzoate). Over a period of several weeks, groups of 3- to 5-day-old females were starved of fructose overnight and subsequently membrane fed on defibrinated whole horse blood (Tissue Culture Laboratories Ltd) containing 0.5mmol l^{-1} ATP added as a phagostimulant (Galun *et al.* 1984). 1.5ml of blood was pipetted onto a 5cm diameter piece of textured glass and covered with double-stretched Parafilm membrane. The membrane was placed over a container of adults and covered with a bowl of 'hand hot' water. Mosquitoes were allowed to feed for 20–30min, based on an assumed normal feeding time of 2.5–19.5min (Reisen and Emory, 1976), and then immobilised immediately on ice for dissection.

Tissue preparation

Midguts of engorged females were dissected free of other tissues and the blood meals were washed out with dissecting buffer (50mmol l^{-1} Trizma base, pH7.8 at 20˚C). The midgut tissue was pooled in 1.5ml Eppendorf tubes containing dissecting buffer and was frozen at −80˚C. Dissection time of one group of mosquitoes never exceeded 3h after feeding. When a total of 1000 midguts had been collected from a number of dissections, the constituent frozen preparations were thawed and centrifuged at 3000***g*** for 1min. The supernatants of dissecting buffer were then removed and the tissue samples resuspended in 50mmol l^{-1} Tris:0.1% Tween 20 (pH7.8 at 20˚C) and pooled on ice. The pooled tissue was homogenised to a uniform suspension in a hand-held glass:glass homogeniser. The suspension was probe-sonicated on ice for 15s at 8–10 μm and then centrifuged at 8000***g*** for 30min (0˚C). The resulting supernatant was centrifuged at 100000***g*** for 2 h (0˚C), and the putative microsomal pellet resuspended in 50mmol l^{-1} Tris to a concentration of 100 midgut equivalents per millilitre. This was then stored at −80˚C in 100 μl samples. Just prior to enzyme assay, frozen tissue samples were thawed and diluted further with 250 μl of 50mmol l^{-1} Tris:0.1% Tween 20. For protein assays, the samples did not undergo this dilution step. There was no significant decrease in activity with storage over the 2 month period of assays, but the freeze–thawing involved in the preparation procedure resulted in up to 30% loss of activity.

Enzyme assays

The enzyme assay was based on that of Peacock (1981) which allows ouabain-sensitive and ouabain-insensitive ATPase activities to be distinguished from each other. The method was adapted for microassay and conditions were optimised for maximum activity. Unless stated otherwise, the standard reaction medium contained 4mmol l^{-1} $MgCl_2$, 120mmol l^{-1} NaCl and 20mmol l^{-1} KCl in 50mmol l^{-1} Tris with 0.5mmol l^{-1} EGTA (pH7.3±0.05 at 20˚C). For each assay a minimum of two 'test' wells containing reaction medium alone, two 'ouabain' wells containing reaction medium with 10mmol l^{-1} ouabain and two control wells were used. Initially, some of the assays were performed on smaller tissue preparations and without a ouabain-inhibited comparison, giving an indication of total enzyme activity. In these assays, where Na^+ and/or K^+ concentrations were varied and the ouabain-insensitive fraction was unaffected by the ionic changes, the total enzyme activity was a valid representation of the ouabain-

sensitive activity. In each well, 73 μl of reaction medium, with or without ouabain, was added to 7 μl of 48mmol l^{-1} Tris-ATP (made up in 50mmol l^{-1} Tris, pH6.5 at 20˚C). 20 μl of tissue preparation were added to 'test' and 'ouabain' wells, giving a final well pH of 7.3 and a reduction in final molarity of ATP and reaction medium components to 73 % of their prepared molarities. The plates were kept on ice until tissue had been added to all wells, then the reaction was allowed to run for 1h at 37˚C. The plates were then held on ice and 100 μl of stopping solution (1:1, 1% Lubrol PX in water:1% ammonium molybdate in 0.9mol l^{-1} H_2SO_4) was added to each well. 20 μl of tissue preparation was then added to the control wells and the plates were left at room temperature for 10min for colour development. The yellow colour produced in the wells was proportional to the amount of inorganic phosphate released from the hydrolysis of ATP during the enzyme reaction (Atkinson *et al.* 1973). The absorbance at 390nm was determined using a Bio-Rad ELISA plate reader.

Ionic variations

Reaction conditions were varied for each assay. For the pH profile a mixed biological buffer of Trizma, Bis-Tris and Capso [3-(cyclohexylamine)-2-hydroxy-1-propanesulphonic acid] was used to stabilise the pH from 5.7 to 10.5, a range wider than was possible with the individual constituent buffers. Where K^+ concentration was varied, the Na^+ concentration was maintained at 73mmol l^{-1}. Where Na^+ concentration was varied, K^+ concentration was maintained at 14.6mmol l^{-1}. Where Na^+ and/or K^+ concentration were varied, the concentrations of all other salts were maintained as for the standard assay and overall ionic strength was standardised at 180mmol l^{-1} using choline chloride. For all other assays, all salts except the one being varied were maintained at the standard assay concentrations. In all except the calcium assays, EGTA, a more efficient Ca^{2+} chelator than EDTA, was used to remove Ca^{2+} from the system to optimise activity further (Knudsen and Johansen, 1989). Ca^{2+} concentration was varied by adding 0–40mmol l^{-1} $CaCl_2$ to the assay medium and EGTA was omitted from the assay. Total osmolarity is not considered to be critical to the functioning of the Na^+/K^+-ATPase in biochemical assays (Bonting, 1970). All solutions were made up in deionised water and all inorganic salts were analytical reagent grade. Tris–ATP was vanadium free and together with the Trizma, Bis-Tris, Capso, Lubrol PX and Tween 20 was supplied by Sigma Chemical Company.

Protein assay

In the standard Bio-Rad protein assay (Bradford, 1976), 100 μl of stored tissue was diluted in 700 μl of phosphate-buffered saline. 200 μl of Coomassie Brilliant Blue G-250 dye was added and the colour allowed to develop for 15min. Triplicate samples were then transferred to a 96-well ELISA plate in 100 μl samples and the colours were read at 600nm using a Bio-Rad ELISA plate reader. Bio-Rad bovine γ-globulin was used as a standard at dilutions of 0–40 μg ml^{-1}. The mean protein concentration of the tissue samples was 17±2.76 μg ml^{-1}.

Inorganic phosphate

100 μl samples of a range of concentrations of KH_2PO_4 were loaded into the wells of a 96-well plate and 100 μl of stopping solution was then added to each well. After a 10min development time the plate was read at 390nm. This was used to plot a standard phosphate curve.

Blood meal contamination

The amount of potential blood meal contamination in the assay was assessed using haemoglobin as a marker. A simple comparison of ATPase activities in guts with or without blood meals removed was not possible as the haemoglobin interfered with the phosphate optical density readings for the assay. 10 midguts containing blood meals (representing the maximal degree of possible contamination), 10 midguts with blood meals removed and 10 midguts from unfed mosquitoes were each dissected into separate Eppendorf tubes containing 200 μl of dissecting buffer. The samples were sonicated for 15s to ensure release of haemoglobin into solution, then a further 800 μl of dissecting buffer was added to each. Three 200 μl aliquots of each sample were then transferred to a 96-well ELISA plate and absorbances at 412nm were recorded (Feldmann *et al.* 1990). The mean absorbance readings of cleaned, fed midguts was 1.2±0.13% of the mean of absorbances for uncleaned, fed midguts (mean=0.745), but was not statistically different from the levels found in unfed midguts (P=0.895). Blood meal contamination of the samples used in all assays was therefore assumed to be negligible, indicating that all ATPase activity measured was of mosquito origin.

Statistics

Each assay was performed in triplicate. The readings in ‘test’ and ‘ouabain’ wells were corrected with respect to control wells to take into account any contaminating inorganic phosphate and any other background absorbance. Errors were calculated as follows: the mean of the control readings was subtracted from each individual ‘test’ and ‘ouabain’ reading. Mean and standard error for each individual set of data points were calculated from these converted values. Ouabain-sensitive Na^+/K^+-ATPase activity was calculated as the difference in activity between corrected ‘test’ and ‘ouabain’ wells and errors were calculated as above, i.e. the mean of ‘ouabain’ readings for one data point was subtracted from each individual ‘test’ reading. Mean and standard error were then calculated using the converted data. This was a relatively simple way of calculating errors on data from which individual readings could not be paired directly. Specific activities were calculated for each data point using the conversion factors obtained from both the standard phosphate and standard protein curves. The Michaelis constant (K_m) and maximal rate of activity (V_{max}) values were calculated using the statistical method of Wilkinson (1961).

Results

Ouabain sensitivity

Enzyme activities determined by the comparative assay demonstrated the ionic requirements and ouabain sensitivity of the total preparation (Fig. 1). Excess ouabain

($7.3 mmol\,l^{-1}$) failed to inhibit 11.1±0.8% of the maximum activity. This ouabain-insensitive fraction was equivalent to the enzyme activity in the presence of Mg^{2+} and K^+ (P=0.733), but was statistically slightly less than the activity in the presence of Mg^{2+} with

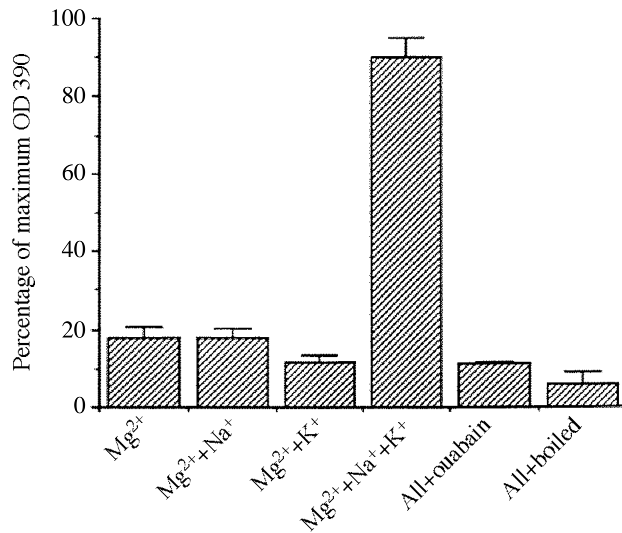

Fig. 1. The change in total ATPase activity (expressed as a percentage of the maximum) in the midgut of *Anopheles stephensi* under various ionic conditions. Mg^{2+} ($3 mmol\,l^{-1}$), Na^+ ($73 mmol\,l^{-1}$), K^+ ($14.6 mmol\,l^{-1}$), ouabain ($0.73 mmol\,l^{-1}$) and boiled enzyme were present in the assays as indicated. Maximum OD 390 corresponded to a specific activity of $2.69\,\mu mol\,P_i\,mg^{-1}\,protein\,min^{-1}$. The method of calculating errors for all figures is described in the text.

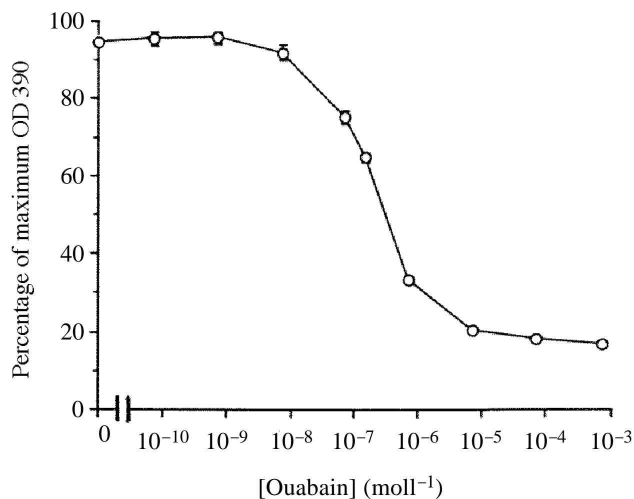

Fig. 2. Dose-dependent inhibition by ouabain of the total ATPase activity in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of $3.23\,\mu mol\,P_i\,mg^{-1}\,protein\,min^{-1}$. Where error bars are not seen, they are smaller than the symbols.

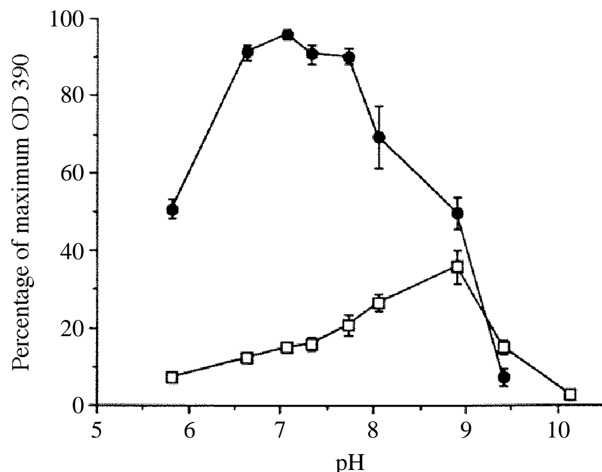

Fig. 3. The effect of pH on ouabain-sensitive (●) and ouabain-insensitive (□) ATPase activities in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of 2.28 μmol P_i mg^{-1} protein min^{-1}. Where error bars are not seen, they are smaller than the symbols.

or without Na^+ (P=0.046 and 0.014, respectively). After boiling the tissue preparation prior to assay, 5.9±2.9% of the maximum absorbance remained. In the ouabain dose–response curve assays, the ATPase activity was inhibited by ouabain in a dose-dependent fashion at concentrations above 10^{-8} mol l^{-1}. Maximum inhibition of Na^+/K^+-ATPase occurred at ouabain concentrations above 10^{-4} mol l^{-1} (10^{-5} mol l^{-1} to 10^{-3} mol l^{-1}, P<0.005, 10^{-4} mol l^{-1} to 10^{-3} mol l^{-1}, P=0.084). The IC_{50} was $4.5\times10^{-7}\pm0.3\times10^{-7}$ mol l^{-1} (Fig. 2). No significant enhancement of activity at 10^{-10} mol l^{-1} ouabain compared to activity without ouabain was observed (P=0.777), although a slight increase was observed. In these ouabain dose–response curve assays, 16.5% of activity remained uninhibited at 0.73 mmol l^{-1} ouabain, while boiling the samples resulted in complete removal of any activity above background (data not included). This indicated that the activity due to ouabain-insensitive ATPase was at least 5.9% and as much as 16.5% of the total activity in the mosquito midgut.

pH sensitivity

Clear differences in pH characteristics were observed between the ouabain-sensitive and ouabain-insensitive enzyme fractions (Fig. 3). The ouabain-sensitive enzyme was optimally active at pH 7.2, while the ouabain-insensitive activity peaked at pH 8.9. The pH values corresponding to regions of greater than 80% activity in the two fractions were mutually exclusive, ranging between pH 6.4 and 7.8 for the ouabain-sensitive ATPase and between pH 8.2 and 9.1 for the ouabain-insensitive activity. All subsequent assays were performed at pH 7.3.

Temperature sensitivity and activity over time

Estimates of the optimum temperatures for the enzyme activities were obtained by

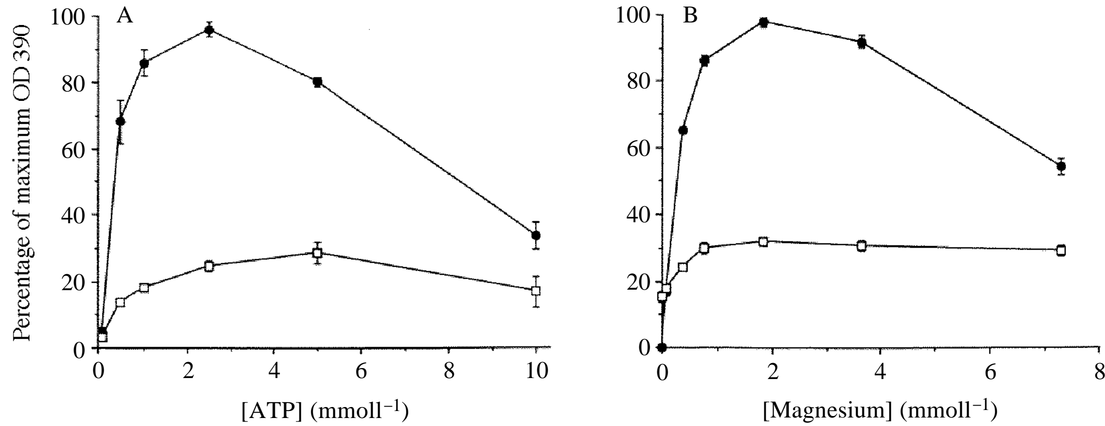

Fig. 4. (A) Effect of Tris-ATP concentration on ouabain-sensitive (●) and ouabain-insensitive (□) ATPase activities in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of 2.15 μmol Pi mg^{-1} protein min^{-1}. (B) Effect of Mg^{2+} concentration on ouabain-sensitive (●) and ouabain-insensitive (□) activities in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of 2.32 μmol Pi mg^{-1} protein min^{-1}. Where error bars are not seen, they are smaller than the symbols.

Table 1. *Kinetic characteristics of ouabain-sensitive and ouabain-insensitive* Na^+/K^+*-ATPase from the midgut of* Anopheles stephensi

	Ouabain-sensitive			Ouabain-insensitive		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
ATP	0.33±0.04	2.40±0.09	7.27	0.54±0.08	0.66±0.05	1.22
Mg^{2+}	0.34±0.03	2.60±0.09	7.65	0.07±0.03	0.74±0.02	10.57
Na^+	6.47±0.01	2.57±0.03	0.40	–	–	–
K^+	0.72±0.02	2.23±0.05	3.09	–	–	–

Michaelis constant (K_m) values are given in mmol l^{-1} of each ion tested and were calculated according to Wilkinson (1961) from three replicates of each assay.

Maximum rates of activity (V_{max}) are given as specific activities (μmol P_i mg^{-1} protein min^{-1}) calculated from the percentage of the maximal OD 390 readings for each assay. The specific activities corresponding to the OD 390 readings are given in the figure legend for the appropriate curves.

V_{max}/K_m is an indication of the catalytic constant (K_{cat}) of the enzymes with each ion.

running the assay at 4, 21, 37 and 60˚C. Both ouabain-sensitive and ouabain-insensitive enzyme fractions were active maximally at 37˚C. All subsequent incubations were therefore performed at 37˚C.

The time dependencies of the ATPases were determined by recording the enzyme activities over increasing incubation periods up to 2h (data not shown). The reaction of the ouabain-sensitive enzyme was linear to 1h (correlation coefficient, r=0.991–0.996); subsequently, there was some deviation from linearity of ouabain-sensitive activity (correlation to 2h, r=0.947). The reaction of the ouabain-insensitive fraction remained linear up to 2h. Subsequent assays were continued with 1h incubation times.

ATP and Mg^{2+} dependence

Increasing the ATP concentration resulted in a typical substrate reaction profile for the ouabain-sensitive enzyme, with a peak activity at 2.5 mmol l^{-1} ATP, and marked substrate inhibition up to 10 mmol l^{-1} (Fig. 4A). A half-maximal activation concentration (K_m) of 0.33±0.04 mmol l^{-1} and V_{max} of 2.4±0.09 μmol P_i mg^{-1} protein min^{-1} were calculated, giving a V_{max}/K_m ratio of 7.27 (Table 1). The ouabain-insensitive fraction also demonstrated an ATP dependence, with a peak of activity at 5 mmol l^{-1} ATP, a K_m of 0.54±0.08 mmol l^{-1} and V_{max} 0.66±0.05 μmol P_i mg^{-1} protein min^{-1}, and a V_{max}/K_m ratio of 1.22.

The Mg^{2+} dependency of the ouabain-sensitive enzyme was similar to that of ATP dependency, with peak activity observed at 1.9 mmol l^{-1} Mg^{2+} and inhibition at higher concentrations (Fig. 4B). The K_m and V_{max} were 0.34±0.03 mmol l^{-1} and 2.6±0.09 μmol P_i mg^{-1} protein min^{-1}, respectively, giving a V_{max}/K_m ratio of 7.65 (Table 1). The ouabain-insensitive fraction showed an increase in activity from 15.4% at 0 mmol l^{-1} Mg^{2+} to 30% at 1 mmol l^{-1} Mg^{2+}. At higher concentrations activity remained at a similar level and no inhibition was observed. The K_m and V_{max} values for the ouabain-insensitive activity were 0.07±0.03 mmol l^{-1} and 0.74±0.02 μmol P_i mg^{-1} protein min^{-1}, respectively, with a V_{max}/K_m ratio of 10.57. There was a 1.3:1 ATP:Mg^{2+} ratio of optimal concentrations for the ouabain-sensitive enzyme.

Dependence on Na^+ and K^+

The ouabain-sensitive enzyme showed a marked dependence on Na^+ concentration, with a sharp increase in activity from less than 10% at 0 mmol l^{-1} to almost 80% at 15 mmol l^{-1} Na^+, and a more gentle but steady increase from 30 to 120 mmol l^{-1} (r=0.980) (Fig. 5A). The activity of the ouabain-insensitive fraction was completely unaffected by changes in Na^+ concentration (between points, $P>0.4$), so values for K_m and V_{max} could not be calculated (Table 1). The K_m and V_{max} values were 6.47±0.01 mmol l^{-1} and 2.57±0.03 μmol P_i mg^{-1} protein min^{-1} for the ouabain-sensitive activity, respectively, with a V_{max}/K_m ratio of 0.40.

The effect of K^+ on the ouabain-sensitive enzyme was demonstrated by a rapid rise in activity from 8% at 0 mmol l^{-1} to 85% at 7 mmol l^{-1}, after which the activity increased less steeply to 15 mmol l^{-1} and then plateaued (Fig. 5B). K_m and V_{max} were 0.72±0.02 mmol l^{-1} and 2.23±0.05 μmol P_i mg^{-1} protein min^{-1} for ouabain-sensitive activity, respectively, with a V_{max}/K_m ratio of 3.09. Again, no effect on activity of the ouabain-insensitive fraction was observed with increasing K^+ concentration ($P>0.7$), and no K_m or V_{max} could be calculated (Table 1).

Simultaneously changing Na^+ and K^+ concentrations, whilst maintaining the total ionic strength constant, caused a 90% loss of total enzyme activity when K^+ was completely absent and an 84% loss of activity when Na^+ was completely absent (Fig. 5C). The increase in activity to 100 mmol l^{-1} Na^+ was less rapid than when Na^+ concentration alone was adjusted, but the increase in activity from 0 to 20 mmol l^{-1} K^+ was similar to that observed when adjusting K^+ concentration alone. A plateau of maximal activation (P=0.227) occurred when the Na^+ concentration was in the range 90–120 mmol l^{-1} with

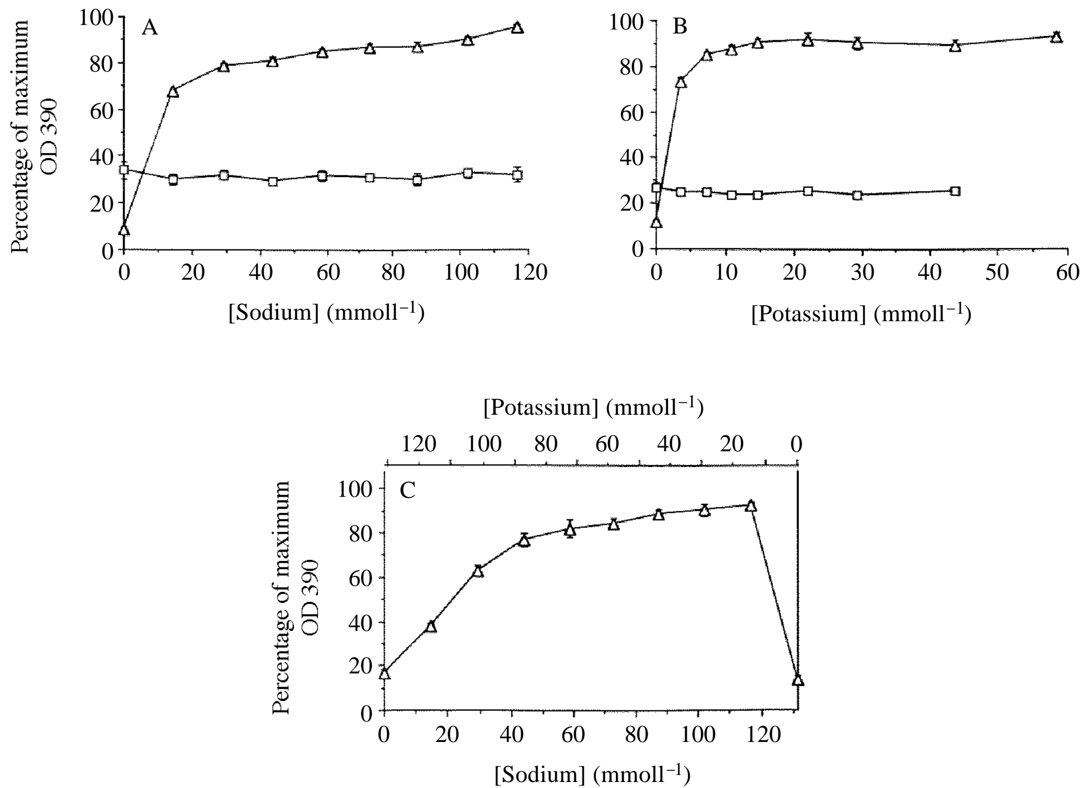

Fig. 5. (A) Effect of Na^+ concentration on total (△) (initial assays) and ouabain-insensitive (□) (follow-up assays) activities in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of 2.69 μmol P_i mg^{-1} protein min^{-1} for total ATPase activity and 2.44 μmol P_i mg^{-1} protein min^{-1} for the differential assays. (B) Effect of K^+ concentration on total (△) (initial assays) and ouabain-insensitive (□) (follow-up assays) activities in the midgut of *Anopheles stephensi.* Maximum OD 390 corresponded to a specific activity of 2.44 μmol P_i mg^{-1} protein min^{-1} for total ATPase activity and 2.49 μmol P_i mg^{-1} protein min^{-1} for the differential assays. (C) Effect of a simultaneous increase in Na^+ concentration and decrease in K^+ concentration on total ATPase activity in the midgut of *Anopheles stephensi.* Maximum OD 390 corresponded to a specific activity of 1.25 μmol P_i mg^{-1} protein min^{-1}. Where error bars are not seen, they are smaller than the symbols.

K^+ concentration between 12 and 42 mmol l^{-1}. Outside this range, the activity was less than maximal.

Effect of calcium

The ouabain-sensitive ATPase was inhibited by Ca^{2+} in a dose-dependent way. The enzyme was not significantly affected by Ca^{2+} in the concentration range 0–0.1 mmol l^{-1} (from *t*-tests, P=0.568). However, 1 mmol l^{-1} Ca^{2+} reduced the activity to 54% of the maximum and complete inhibition occurred at 4 mmol l^{-1} Ca^{2+} (Fig. 6). The IC_{50} was

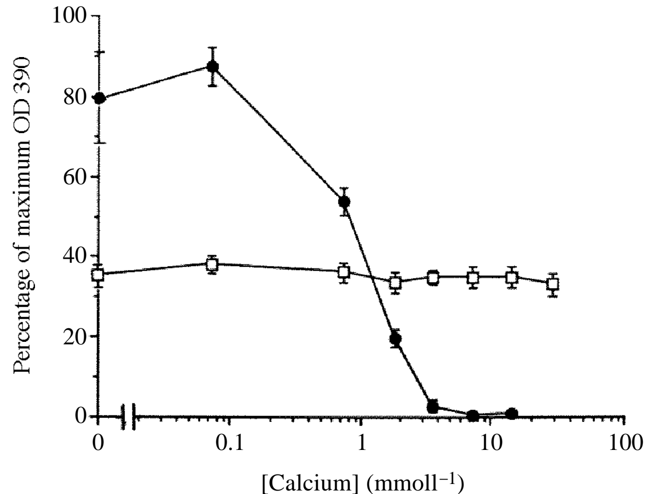

Fig. 6. Effect of increasing Ca^{2+} concentration on ouabain-sensitive (●) and ouabain-insensitive (□) activities in the midgut of *Anopheles stephensi*. Maximum OD 390 corresponded to a specific activity of 2.45 μmol P_i mg^{-1} proteinmin^{-1}.

1.11±0.07mmoll^{-1}. The ouabain-insensitive enzyme fraction was unaffected by increasing the concentration of Ca^{2+} up to 30mmol l^{-1} (0.1–30mmoll^{-1}, *P*=0.215).

Discussion

The present study is the first to have demonstrated ouabain-sensitive Na^+/K^+-ATPase activity in the midgut of *A. stephensi* and shown it to be a major ATPase in this tissue. However, a significant proportion (up to 16.5%) of the ATPase activity measured was insensitive to ouabain. The comparative assays of ionic and ouabain sensitivities clearly illustrated the presence of a ouabain-sensitive ATPase, as well as demonstrating the effects of various ions on the ouabain-sensitive and ouabain-insensitive enzyme activities. Any activating effects of the monovalent cations on the ouabain-insensitive activity could have masked the true activity of the ouabain-sensitive Na^+/K^+-ATPase in the standard differential assay (Anstee and Bowler, 1984). The present study demonstrated a complete inhibition by ouabain of the Na^+- and K^+-dependent enzyme and negligible effect of Na^+, K^+ and Ca^{2+} on the remaining ATPase.

Another clear difference in biochemical characteristics between the ouabain-sensitive and ouabain-insensitive enzymes was observed in the pH assay. The pH profiles for the two fractions were similar to those described for the standard differential Na^+/K^+-ATPase assay in other tissues (Anstee and Bowler, 1984; Bonting, 1970; Rutti *et al.* 1980; Schin and Kroeger, 1980). The standard assay pH of 7.2–7.3 provided optimal pH conditions for the ouabain-sensitive enzyme, whereas at this pH the ouabain-insensitive fraction was 30% active and represented only 15% of the maximum activity. As the enzyme is probably located in the basolateral plasma membrane (Fogg *et al.* 1991), haemolymph rather than midgut lumen conditions would be expected to affect *in vivo* activity. In the

absence of relevant haemolymph pH measurements it is not possible to discuss the physiological effects of pH on the Na^+/K^+-ATPase. However, the different pH optima suggest that the two enzymes are active in different physiological environments (see below).

The peak of activity at pH9 for the ouabain-insensitive fraction, together with its total lack of dependence on Na^+, K^+ or Ca^{2+}, is consistent with it being an F-type mitochondrial Mg^{2+}-ATPase. This enzyme uses Mg^{2+} as a reaction cofactor, which results in activation by Mg^{2+} in the assay. The mitochondrial inner membranes on which the Mg^{2+}-ATPase resides are commonly found in microsomal membrane preparations (Dhalla and Zhao, 1988). The relatively low K_m values shown in the mosquito for ATP and Mg^{2+} for both ouabain-sensitive and ouabain-insensitive enzymes demonstrate high affinities of both the enzymes for these reagents as either substrates or catalysts. The K_m/V_{max} values are an indication of the catalytic constants, which demonstrate the rate of catalysis of substrate by the enzymes. The ouabain-sensitive enzyme shows a high turnover of ATP, and both enzyme fractions show a high turnover of Mg^{2+}. This reinforces the suggestion that the ouabain-insensitive activity is a Mg^{2+}-dependent mitochondrial ATPase and that the ouabain-sensitive fraction shows classic Mg–ATP catalysis. The Mg–ATP complex is the true enzyme substrate for the Na^+/K^+-ATPase (Hexum *et al.* 1970; Robinson, 1983) such that Mg^{2+} cannot be eradicated from the assay and, therefore, the mitochondrial ATPase will always be active. The optimal 1:1.3 ratio of Mg:Tris–ATP observed suggests that the Mg–ATP complex was indeed formed in these assays.

The ouabain inhibition profile was qualitatively similar to that described for Na^+/K^+-ATPase from other insect tissues, such as cockroach rectal epithelium (Tolman and Steele, 1976) and antennae (Norris and Cary, 1981) and *Chironomus thummi* salivary gland (Schin and Kroeger, 1980). In most tissues the IC_{50} for ouabain falls between 10^{-5} and 10^{-7} mol l^{-1} (Skou, 1988), with relatively high IC_{50} values of around 10^{-6} being reported for Malpighian tubules of *Locusta migratoria* (Anstee and Bell, 1975) and anterior midgut of *Glossina morsitans* (Peacock, 1981). In both of these insects, fluid secretion was dependent on the presence of an active Na^+/K^+-ATPase. The IC_{50} of 4.5×10^{-7} mol l^{-1} in *A. stephensi* indicates a Na^+/K^+-ATPase with similar ouabain sensitivity in the mosquito midgut, and is consistent with a potential role for ouabain-sensitive Na^+/K^+-ATPase in fluid secretion from the mosquito midgut.

The specific inhibition of Na^+/K^+-ATPase by ouabain is a useful diagnostic phenomenon. Isoforms of the enzyme show different sensitivities to inhibition by ouabain and other cardiac glycosides and, within a species, a direct correlation between inhibitor affinity and isoform type has been found (Price and Lingrel, 1988). The IC_{50} of 0.45 μmol l^{-1} for the mosquito ouabain-sensitive ATPase suggests the presence of an isozyme with a high ouabain affinity (Anstee *et al.* 1986; Sweadner, 1989). No significant enhancement of Na^+/K^+-ATPase activity at low concentrations of ouabain was observed, unlike observations in several mammalian tissues (Bonting, 1970). The absence of any biphasic effect in the linear portion of the ouabain inhibition curve (i.e. more than one line gradient generated from the data, causing a shoulder on the curve), could suggest the absence of isoforms within the mosquito midgut (Marks and Seeds, 1978; Sweadner,

1979, 1989). Only one isoform has been reported to be present in *Drosophila melanogaster*, the genome of which is thought to contain only one gene encoding the α-subunit (Lebovitz *et al.* 1989). This is in contrast to mammalian tissues, in which multiple isoforms are encoded by different genes (Shull and Lingrel, 1986).

The biochemical characteristics of the ouabain-sensitive activity were qualitatively similar to those from several tissues characterised previously. The specific activity of the ouabain-sensitive Na^+/K^+-ATPase in *A. stephensi* midgut was comparable to that reported for housefly head (Jenner and Donnellan, 1976), *Rhodnius prolixus* (Hemiptera) ovarian follicle (Ilenchuk and Davey, 1982), *Periplaneta americana* (Orthoptera) antennae and rectal epithelia (Norris and Cary, 1981; Tolman and Steele, 1976) and *Amblyomma hebraeum* (Acarina) salivary gland, in which Na^+/K^+-ATPase is thought to be important for fluid secretion (Kaufman *et al.* 1976; Rutti *et al.* 1980). It was, however, apparently tenfold higher than the activity reported for *G. morsitans* anterior midgut (Peacock, 1981, 1982). The methods of preparation may affect the specific activity of Na^+/K^+-ATPase as well as having a fundamental influence on its kinetic properties (Sweadner, 1989). Preparative differences are certainly seen in the considerable variation (1–80 mmol l^{-1} for K^+ and 4.5–100 mmol l^{-1} for Na^+) in reported K_m values from different tissues (Bonting, 1970; Anstee and Bowler, 1984). The K^+ K_m value for the *A. stephensi* ouabain-sensitive enzyme correlated most closely in mammals to that of rat kidney (K_m=0.7 mmol l^{-1}; Bonting, 1970) and in insects to that of housefly head and locust Malpighian tubules (K_m=1 mmol l^{-1}; Anstee and Bowler, 1984). The Na^+ K_m was most similar to that of rat liver in mammals (K_m=6–8 mmol l^{-1}; Bonting, 1970) and to that of cockroach antenna amongst the insects (K_m=6.3 mmol l^{-1}; Anstee and Bowler, 1984). Given the preparative variations, it is not possible to conclude definite similarities from this comparison. However, the kinetic properties of the mosquito enzyme appear to match most closely those of the rat kidney. Taking the ouabain data into consideration, this similarity would be surprising as the rat kidney α1 isozyme has been shown to have a low ouabain affinity (Sweadner, 1989). In the present study, ion concentrations used in the assays may appear to be lower than previously reported values. However, our given values are the molarities in the assay wells *after* addition of ATP and enzyme homogenate, and this accounts for some quantitative dissimilarities.

In the mosquito, the ouabain-sensitive enzyme showed a higher affinity for K^+ (K_m=0.72) than for Na^+ (K_m=6.47) and, together with the results of the effects of simultaneous increase/decrease in the concentrations of the monovalent cations, this compares closely to similar work with mammalian tissues (Skou, 1988). Even at low concentrations, K^+ activates at the extracellular sites when cytoplasmic sites are saturated with Na^+ (right-hand side of Fig. 5C). Conversely, Na^+ activates the enzyme at the cytoplasmic sites when extracellular sites are saturated with K^+ (left-hand side of Fig. 5C), although the increase in activity was suppressed compared to the situation when Na^+ concentration alone was changed and K^+ concentration was not saturating (cf. Fig. 5A). This may indicate an inhibitory effect of K^+ at high concentrations. Choline, which was present in some of the assays, may be antagonistic to K^+ (Esmann, 1988), but no obvious antagonistic effects were observed when comparing the present data with other published results.

The *in vivo* concentrations of ions in the mosquito midgut epithelial cells, haemolymph and midgut lumen remain to be clarified. However, the physiological ion concentrations in related tissues could give some indication of how the mosquito Na^+/K^+-ATPase may be functioning in the midgut *in vivo*. Non blood-feeding insects have varying intracellular concentrations according to the nutritional state of the insect (Gupta *et al.* 1980), and such variations would be expected to occur after blood feeding, which results in a sudden and massive increase in midgut water and salt concentrations. If the intracellular ion concentrations in the *A. stephensi* midgut are similar to those in the rectal papillae (normally a water-regulatory region in insects) of *Calliphora erythrocephala* (10–20 $mmol\,l^{-1}$ Na^+ and 115 $mmol\,l^{-1}$ K^+), the *in vivo* activation of the mosquito Na^+/K^+-ATPase by Na^+ would be 30–40% maximal. At extracellular ion concentrations of 10 $mmol\,l^{-1}$ K^+ and 120 $mmol\,l^{-1}$ Na^+, the K^+ activation of the Na^+/K^+-ATPase would be 70% maximal. Under these conditions, the Na^+/K^+-ATPase transport system in the mosquito midgut would operate at about 20–30% maximum (Skou, 1988).

Inhibition of Na^+/K^+-ATPase by Ca^{2+} has been well documented (Apell and Marcus, 1986; Hagane *et al.* 1989; Knudsen and Johansen, 1989) and results from competition by Ca^{2+} for Mg^{2+} binding sites on the enzyme. Ca^{2+} may also occupy the binding site for K^+ on the isolated enzyme, but is not a utilisable substrate (Vasallo and Post, 1986). Although Ca^{2+} concentrations of 1.5–51 $mmol\,l^{-1}$ in insect haemolymph have been reported, it was suggested that cytosolic levels were 0.1–0.3 $mmol\,l^{-1}$ (Taylor, 1986) and cytosolic rather than extracellular Ca^{2+} would compete for Mg^{2+} binding sites. At *in vivo* levels of 0.1–0.3 $mmol\,l^{-1}$ Ca^{2+} little or no inhibition would occur.

The midgut epithelium of mosquitoes may be a site of major ion and water transport (Billingsley, 1990*a*; Houk *et al.* 1986*a*,*b*). The results of the present study are consistent with a ouabain-sensitive, membrane-associated Na^+/K^+-ATPase having a major role in these processes in the midgut of *A. stephensi*. Other preliminary results indicate that Na^+/K^+-ATPase activity in the midgut is triggered by blood meal ingestion. The physiological and functional role of the enzyme, its control by exogenous (Nijhout and Carrow, 1978) and endogenous (Williams and Beyenbach, 1983, 1984) factors and the potential of the enzyme as an anti-mosquito target site remain to be investigated.

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