Properties of the V-type ATPase from the excretory system of the usherhopper, *Poekilocerus bufonius*

Z.I.A. Al-Fifi a,*, A. Al-Robai a, S.M. Khoja b

*King Abdulaziz University, Faculty of Science, Department of Biological Sciences, P.O. Box 80203, Jeddah 21589, Saudi Arabia
bKing Abdulaziz University, Faculty of Science, Department of Biochemistry, P.O. Box 80203, Jeddah 21589, Saudi Arabia

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Abstract

The bafilomycin A 1 and N-ethylmaleimide (NEM)-sensitive (V-type) ATPase was partially purified from the apical membrane-rich fractions of excretory system (Malpighian tubules and hind gut) of *P. bufonius*. Enzymatic activity was inhibited by bafilomycin A 1 (IC 50 = 1.3 nM) and NEM (IC 50 = 10.1 µM). The V-type ATPase activity is confined to the apical membrane fraction, while the activity of Na\(^+\)/K\(^+\)-ATPase forms the major part of the basal membrane fraction. The optimal pH required for maximal activity of V-type ATPase was pH 7.5. The effect of 30 mM of various salts on ATPase activity was investigated. NaCl and KCl caused increases of 175% and 184%, respectively. Other chloride salts also caused an increase in activity in the following ascending order: RbCl, LiCl, choline Cl, NaCl, KCl and tris-HCl. The activity of V-type ATPase was stimulated by a variety of different anions and cations, and HCO\(_3^-\) was found to be the most potent cationic activator of ATPase activity. The present results show that the properties of V-type ATPase of *P. bufonius* are similar to those reported for other insect tissues. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Insect; *Poekilocerus bufonius*; Excretory system; V-type ATPase; Bafilomycin A1; N-ethylmaleimide

1. Introduction

It has generally been accepted that the movement of ions into the epithelial cells occurs via Na\(^+\)/K\(^+\)-pump in Malpighian tubules (see reviews by Berridge (1967); Berridge and Oschman (1969); Maddrell (1971, 1977, 1980); Anstee and Bowler (1984). The influx of K\(^+\) and the efflux of Na\(^+\) are mediated by active transport. In the majority of the studied tissues, this exchange between the ions is mediated by the Mg\(^2+\)- dependent, (Na\(^+\)/K\(^+\))-stimulated ATPase (Skou, 1957). Further studies revealed that the enzyme is ubiquitous in animal cells where its properties and location have been established (Skou, 1965; Schuurmans Stekhoven and Bonting (1981); Skou and Esmann (1992); Al-Fifi et al. (1998a); Emery et al. (1998).

It has been fully understood that ouabain is specific inhibitor of the Na\(^+\)/K\(^+\)-ATPase activity in a number of different species of animals (Skou, 1965; Keynes, 1973; Anstee and Bowler, 1984; Al-Robai et al., 1992; Skou and Esmann, 1992; Linton and O'Donnell (1999). It is well established that the Na\(^+\)/K\(^+\)-ATPase is restricted to the basal cell membrane and upon ATP hydrolysis, 3Na\(^+\) is translocated in exchange for 2K\(^+\). Berridge and Oschman (1969) proposed that the basal electrogenic Na\(^+\)/K\(^+\)-ATPase is responsible for the cytoplasm of most cells having a high (100–160 mM) K\(^+\) concentration, but relatively low (3–30 mM) Na\(^+\) concentration (Ernst et al., 1980; Schuurmans Stekhoven and Bonting, 1981; Anstee and Bowler, 1984; O'Donnell and Maddrell (1984); Lechleitner and Phillips (1988); Lebovitz et al. (1989); Fogg et al. (1991); Skou and Esmann (1992).

The activity of V-type H\(^+\)-ATPase has been measured at the apical membrane of the insect epithelial cells from different species (Harvey, 1992; Klein et al., 1991; Garayoa et al., 1995; Al-Fifi et al., 1998a; Wieczorek et al., 1999b; Beyenbach et al., 2000; O'Donnell and Spring (2000). It has been stated that the V-type H\(^+\)-ATPase creates a proton motive force (PMF) across the apical membrane (Schweikl et al., 1989; Wieczorek et
Al-Fifi et al., 1989, 1991, 1999a, 2000; Wieczorek, 1992; Zhang et al., 1994; Harvey et al., 1998). The PMF acts in parallel with a K*/H+ antipporter to affect the active transport of K+ across the apical membrane, which in turn is the driving force for fluid secretion (Harvey et al., 1983; O’Donnell et al., 1996; Hopkin et al., 1999). The activity of the V-type H+ATPase, and hence of cation transport, is controlled hormonally (Davies et al., 1995; Hopkin et al., 1999; Al-Fiﬁ et al., 1998b; O’Donnell and Spring, 2000; Coast et al., 2001). The insect physiologist sees the enzyme as an energizer of plasma membranes; the transmembrane voltage it generates drives nutrient uptake and fluid secretion, in some cases alkalinizing the gut lumen (Harvey et al., 1998; Wieczorek et al., 1999b; Grüber et al., 2001).

It has been reported that insects feeding on plants high in K+ were shown to be insensitive to ouabain (Jungreis and Vaughan, 1977; Moore and Scudder, 1986). The usherhopper, Poekilocerus bufonius, in nature probably feeds exclusively on toxic plants, one of which is Calotropis procera (Euw et al., 1967; Duffey, 1980; Al-Robai et al., 1998). This plant contains cardiac glycosides (Al-Robai et al., 1998) and a minute amount of its latex is toxic to both vertebrates and invertebrates (Detweiler, 1967; Mahmoud et al., 1979a, 1979b; Al-Robai et al., 1995a, 1995b; Al-Robai, 1997).

It has been previously investigated that the activity of Na+/K+-ATPase in the microsomal preparations of the excretory system (Malpighian tubules and hindgut) and mid gut of usherhopper was resistant to inhibition by ouabain (Al-Robai et al., 1990; Al-Robai, 1993). However, the properties of V-type H+ ATPase of the excretory system of usherhopper, Poekilocerus bufonius, have not been previously investigated, hence the present study was undertaken.

2. Materials and methods

Male and female usherhoppers, Poekilocerus bufonius (Klug), were collected from the Gizan area in southwestern Saudi Arabia. They were kept in cages at 28 ± 1 °C, with access to branches of fresh Usher milk-weed, Calotropis procera.

2.1. Preparation of membrane microsomes

Animals were killed by decapitation, and the excretory system, comprising of the Malpighian tubules and the hindgut, was removed and the contents discarded. Tissue from approximately 30 animals was added to 10 ml of ice-cold homogenization medium (250 mM sucrose and 5 mM tris-HCl buffer, pH 7.5). All subsequent steps were carried at 4 °C. Homogenization was performed in a glass homogenizer with a Teflon pestle (clearance 0.1–0.15 mm) with 20 passes of the plunger at 1000 revs/mm. Membrane microsomes were then isolated by differential centrifugation according to the protocol of Rodriguez and Edelman (1979) and Fogg et al. (1991), as described in Al-Fiﬁ et al., (1998a).

2.2. Assay of ATPase activity

The ATPase activity of the isolated membrane fractions was measured according to the method of Al-Fiﬁ et al., (1998). Briefly, each incubation medium, containing 250 µl of an appropriate ionic medium (see below) and 125 µl of membrane preparation, was equilibrated in a waterbath for 15 mins at 35 °C. The assay was initiated by the addition of 125 µl of 12 mM ATP (Tris salt) and run for 30 mins at 35 °C. ATP (Tris salt) concentrations were varied between 0 and 4 mM in assays employed to elucidate the effect of ATP concentration on V-type ATPase activity. 1000 µl of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9 M sulphuric acid was used to halt the reaction (Atkinson et al., 1973), after which the tubes were left for 10 mins at room temperature to allow colour development to occur. Following centrifugation at 10000 rpm for 10 mins, absorbancy was measured at 390 nm. The intensity of the yellow colour developed was proportional to the amount of inorganic phosphate in the assay. Thus, enzyme activity was measured by determining the amount of inorganic phosphate released. The following ionic media were used to assay different ATPase activities (final concentrations):

2.2.1. Assay of Na+/K+-ATPase activity

(1) 4 mM MgCl2; (2) 4 mM MgCl2, 100 mM NaCl, and 20 mM KCl; (3) 4 mM MgCl2, 100 mM NaCl, 20 mM KCl plus 1 mM ouabain. Each medium contained 20 mM imidazole/HCl (pH 7.2). Na+ /K+-ATPase activity was determined as the difference in the amount of inorganic phosphate liberated in the presence of ionic medium 2 and 3.

2.2.2. Assay of V-Type ATPase activity

This was performed as described by Schweikl et al., (1989) by assaying the azide-and orthovanadate-insensitive ATPase activity of the membrane fraction (Al-Fiﬁ et al., 1998a). The ionic media used were: (1) 1 mM MgCl2, 20 mM KCl, 50 mM Tris-MOPS, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 0.5 mM NaN3, 1 mM sodium orthovanadate, 0.3 mg BSA/ml and 0.05% Triton X-100 (pH = 7.5); (2) Medium (1) plus n-ethylmalimide (NEM) (0.0–1.0 mM); (3) Medium (1) plus bafloymycin A1 (0.0–1.0 mM).

Additionally, the effect of various salts on V-type ATPase activity was determined in the presence of 1 mM MgCl2, 5 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM NaN3 and 0.1 mM Na3VO4. The salts used included monovalent chlorides (K+, Na+, Rb+ and Li+) and choline...
chloride, and anion salts of K\(^+\) (SO\(_4^{2-}\), F\(^-\), Br\(^-\), gluconate and NO\(_3^-\)). All the above salts were used at a concentration of 30 mM.

V-type ATPase activity was also examined over a pH range of 6.0 to 10.0. The reaction medium used contained 50 mM Tris-MOPS, 20 mM KCl, 1 mM EGTA, 0.5 mM NaN\(_3\), 0.1 mM Na\(_3\)V\(_4\), 0.05% Triton X-100 and 0.3 mg ml\(^{-1}\) BSA (pH 7.5), allied to a 30 mM Bis-Tris Propane buffer system.

Appropriate controls were used to determine the extent of non-enzymatic hydrolysis of ATP. All ATPase activities are expressed in nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\).

2.3. Estimation of protein concentration

Protein concentrations were determined according to the method of Lowry et al., (1951), with bovine serum albumin (BSA) fraction V as the standard.

2.4. Reagents

All solutions were prepared in glass-distilled deionised water. All inorganic salts were AnalaR grade or the purest commercially available. Ba\(_2\)fiolomycin A\(_1\) was dissolved in DMSO before adding it to the appropriate solution and the final concentration of DMSO in the experimental solution was < 0.1%. The same concentration of solvent was included in the controls.

3. Results

3.1. ATPase activities in basal and apical membrane fractions

The Na\(^+\)/K\(^+\)-ATPase and V-type ATPase activities in the basal and apical membrane fractions of \(P. \)bufonius are summarized in Table 1. V-type ATPase activity was found to be greatest in the apical membrane fractions, and was 539.8 ± 119.3 nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\). The low activities of Na\(^+\)/K\(^+\)-ATPase and V-type ATPase activities in the apical and basal fractions respectively may be due to contamination.

3.2. Effect of ATP concentration on the Activity of V-type ATPase

Fig. 1 shows the effect of variation in the ATP concentrations on V-type ATPase activity. The assay was carried out in a reaction medium in which ATP (Tris-salt) concentration was varied between 0.0 and 4 mM. There was no enzyme activity in the absence of ATP, whilst maximal activity was observed at a concentration of 1 mM. The shape of the relationship between enzyme activity and ATP concentration was hyperbolic. The V-type ATPase activity at 1 mM ATP was 2108.1 ± 155.7 nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\), and decreased to 1589.70 ± 134.10 nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\) at a concentration of 4 mM.

3.3. Effect of pH on V-type ATPase activity

Fig. 2 shows the change observed in V-type ATPase activity in response to variations in pH. The measured enzyme activity was lowest at pH 6.0, and increased to give maximum activity at pH 7.5 (1879.50 ± 132.30 nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\)). Enzyme activity decreased sharply as the pH was increased above this value. (p < 0.05).

![Fig. 1. Effect of different concentrations of ATP on V-type ATPase activity of P. bufonius excretory system. Enzyme activity expressed as n moles Pi liberated mg protein\(^{-1}\) min\(^{-1}\). (Each point represents mean ± SEM. \(n = 3\)).](image-url)
3.4. Effect of NEM concentration on V-type ATPase activity

The sensitivity of V-type ATPase to NEM is shown in Fig. 3, in which residual activity plotted as a percentage of the inhibited rate against the -log_{10} NEM concentrations. The concentration resulting in 50% inactivation of V-type ATPase activity (IC_{50}) was 10.1 µM. It is clear that V-type ATPase activity in the apical fraction of *P. bufonius* is highly sensitive to NEM.

3.5. Effect of bafilomycin A_{1} concentration on V-type ATPase

Fig. 4 shows the effect of different concentrations of bafilomycin A_{1} (0.0–1.0 mM) on the V-type ATPase. In agreement with earlier studies (reviewed by Forgac, 1989; Nelson, 1992; Nicolson, 1993; Dow, 1994; and Beyenbach (1995), the activity was extremely sensitive to bafilomycin, since a concentration of only 0.1 nM significantly (p<0.05) reduced the V-type ATPase activity in the apical fraction by approximately 23% of that of the controls. 10 nM effected an almost total inhibition of V-type ATPase activity, and the calculated IC_{50} of V-type ATPase activity was 1.3 nM.

3.6. Effect of various salts on the activity of V-type ATPase

The results presented in Table 2 summarize the response of the V-type ATPase to a number of different salts in comparison to the activity of the enzyme in the standard reaction medium (1 mM MgCl_{2}, 5 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM NaN_{3} and 0.1 mM Na_{3} VO_{4}). With the exception of KBr, all the salts
values were obtained by comparing the activity in the presence and absence of medium depended on each salt experiment. Mean values ± SEM.

ATPase is contrast, NO3
trast, NO3

Table 2:
Influence of various salts on V-type ATPase activity of excretory system of P. bufonius

<table>
<thead>
<tr>
<th>Salt (30nM)</th>
<th>Relative specific activity of V-type ATPase (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Salt*</td>
<td>100%</td>
</tr>
<tr>
<td>KCl</td>
<td>183.9 ± 13.1 &lt;0.002</td>
</tr>
<tr>
<td>NaCl</td>
<td>174.9 ± 22.0 &lt;0.01</td>
</tr>
<tr>
<td>Choline Cl</td>
<td>154.1 ± 18.3 &lt;0.02</td>
</tr>
<tr>
<td>RbCl</td>
<td>129.7 ± 13.5 &lt;0.05</td>
</tr>
<tr>
<td>LiCl</td>
<td>138.4 ± 17.6 &lt;0.05</td>
</tr>
<tr>
<td>KHCO3</td>
<td>261.3 ± 31.2 &lt;0.0001</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>241.9 ± 29.1 &lt;0.0001</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>212.5 ± 26.7 &lt;0.001</td>
</tr>
<tr>
<td>KBr</td>
<td>109.1 ± 19.4 n.s.</td>
</tr>
<tr>
<td>K Gluconate</td>
<td>156.3 ± 21.0 &lt;0.05</td>
</tr>
<tr>
<td>KSO4</td>
<td>132.8 ± 27.8 &lt;0.05</td>
</tr>
<tr>
<td>KF</td>
<td>138.2 ±16.7 &lt;0.05</td>
</tr>
<tr>
<td>KNO3</td>
<td>33.6 ± 5.7 &lt;0.0001</td>
</tr>
</tbody>
</table>

* (No salt) Unstimulated ATPase activity obtained in assay medium: 1 mM MgCl2, 5 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM NaN, and 0.1 mM Na2MoO4. The specific activity recorded in this assay medium depended on each salt experiment. Mean values ± SEM. P values were obtained by comparing the activity in the presence and absence of salt using Student’s t-test, (n = 3).

tested had a significant effect on the specific activity of V-type ATPase.

First, the effect of changing the concentrations of a range of monovalent cations (K+, Na+, Rb+, Li+, Choline and Tris) indicates that maximal activation occurred with Tris, and was approximately 213%. The cations were stimulatory in the following sequence; Tris > K+ > Na+ > choline > Li+ > Rb+. K+ had a stimulatory effect of approximately 175%, whilst Rb+ effect was the smallest at approximately 130%.

The effect of the anion salts of K+ (HCO3, SO4, F-, Br-, gluconate and NO3) is also illustrated. HCO3 produced the greatest stimulation of enzyme activity with a value of approximately 261%; in contrast, NO3 was inhibitory to V-type ATPase activity, which was reduced to approximately 34% of the control value. The anions were stimulatory in the following sequence; HCO3 > gluconate = F- = SO4 > Br- > NO3. Br- elicited no effect on ATPase activity, though the sodium salt of HCO3 caused a similar level of stimulation to its potassium salt counterpart.

4. Discussion

The results presented in this study revealed the presence of Na+/K+-ATPase and V-type ATPase in Malpighian tubules and hindgut of P. bufonius. The Na+/K+-ATPase is confined to the basal membrane of epithelial cells of P. bufonius. These results agree well with previous membrane separation works of Fogg et al., (1991) and Al-Fi et al., (1998a) on the Locusta migratoria Malpighian tubules; Linton and O’Donnell (1999) on Drosophila melanogaster Malpighian tubules; Rodriguez and Edelman (1979) on toad bladder epithelium, and with reports that the ouabain-sensitive Na+ pump is restricted to this cell surface in a variety of different tissues from various animal species (Ernst et al., 1980; Anstee and Bowler, 1984; Anstee et al., 1986). Lechleitner and Phillips (1988) also provided biochemical and electron microscopic evidence for the concentration of Na+/K+-ATPase in the basolateral membranes of locust rectal pad epithelium. Subsequent studies on larval midgut of M. sexta (Schweikl et al., 1989; Wieczorek et al., 1989, 1991), and on L. migratoria Malpighian tubules (Al-Fi et al., 1998a) have reported the presence of V-type ATPase activity associated with the isolated apical membrane fraction.

The activity of V-type ATPase was substantially inhibited by NEM (IC50 ~ 10.1 µM), which is in agreement with the reported NEM sensitivity for the enzyme of L. migratoria (Al-Fi et al., 1998a), and M. sexta (Schweikl et al., 1989). NEM is a specific inhibitor of Vacuolar or V-type ATPases when used at appropriate concentrations; V-type ATPases are sensitive to much lower concentrations of NEM (1–2 µM) than the phosphorylated or P-type ATPases (100 µM–1 mM) and the F-type ATPases characteristic of mitochondria, chloroplasts and bacteria are virtually resistant (Forgac, 1989). In the study by Schweikl et al., (1989), 1 µM NEM caused approximately 50% inhibition of enzyme activity and so provided significant evidence that a V-type ATPase may play a part in ion and fluid secretion. Furthermore, this ATPase activity was extremely sensitive to bafilomycin A1 (95% inhibition at ~ 100 nM). Bafilomycin A1, a macroline antibiotic, has been shown to be an extremely potent inhibitor of V-type ATPases (Bowman et al., 1988; Youmans and Barry, 2001). Similar results are reported in L. migratoria (Al-Fi et al., 1998a). There is, therefore, clear evidence that ATPase is present in Malpighian tubules and hindgut of P. bufonius, and that this activity is associated with their apical surface. All the evidences reported yet support the proposed role played by V-type ATPase in ion and fluid transport across the apical membrane of the excretory system of animals (Bertram et al., 1991; Klein, 1992; Klein et al., 1991; Wessing et al., 1993; Dow, 1994; Dijkstra et al., 1994; Wieczorek et al., 1999a; Beyenbach et al., 2000).

Biochemical studies on the V-type ATPase revealed that maximal ATP hydrolysis was obtained at pH 7.5, a value similar to that reported for the enzyme from L. migratoria Malpighian tubules (Al-Fi et al., 1998a), and mammalian brush border (Ait-Mohamed et al., 1986; Wang and Gluck, 1990), but lower than that...
reported for the enzyme from *M. Sexta* midgut (Wiczkorek et al., 1986). Furthermore, the enzyme activity was activated by a variety of both anions and monovalent cations, but it was inhibited by nitrate. These findings, which are in agreement with those reported by Al-Robai et al., (1998a) on the *Locusta migratoria* Malpighian tubules, suggested that it may be non-specific and probably a response to the change in ionic strength. Similar feature of V-type ATPases has been reported from other sources, (O’Neill et al. (1983); Lichko and Okorokov (1984)).

As described previously by Al-Robai et al., (1998), the *P. bufonius* feed on leaves of *Calotropis procera*, which contains high concentrations of toxic cardiac glycosides. The insect ingests, sequesters and concentrates toxic cardiac glycosides in bilobed poison glands. It is already established that the Na\(^+/\)K\(^+\)-transporting ATPase of the excretory system of the usharhopper is tolerant to toxic cardiac glycosides (Al-Robai et al., 1990; Al-Robai, 1993).

The biochemical properties of the V-type ATPase reported in the present study strongly support the existence of a classical enzyme in the Malpighian tubules and hindgut of *P. bufonius*. It is proposed that, in common with other insect secretory epithelia, this enzyme creates a proton motive force across the apical membrane and acts in parallel with a K\(^+/\)H\(^+\) antiporter to affect the active transport of K\(^+\) across the apical membrane (Harvey et al., 1983; Maddrell and O’Donnell, 1992; Weltens et al., 1992; Dow, 1994; Zhang et al., 1994; O’Donnell et al., 1996; Al-Fifi et al., 1998a). The mechanism of ion and fluid secretion in insect Malpighian tubules has been extensively investigated (reviewed by Maddrell, 1971, 1980; Maddrell and O’Donnell, 1992; Nicolson, 1993; Pannabecker, 1995; Beyenbach, 1995). The proposed mechanism suggests that the basal membrane possesses Na\(^+/\)K\(^+\)-ATPase, whereas the apical membrane possesses V-type ATPase. The function of these enzymes involves the regulation of cell volume and metabolism through anion and cation transport (Emery et al., 1998; Al-Fifi et al., 1998a; Wiczkorek et al., 2000).

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