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Cation-stimulated ATPase activity in purified plasma membranes from tobacco hornworm midgut

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Purified goblet cell apical membranes from *Manduca sexta* larval midgut exhibit a specific ATPase activity approx. 20-fold higher than that in the 100 000 × *g* pellet of a midgut homogenate. The already substantial ATPase activity in this plasma membrane segment is doubled in the presence of 20–50 mM KCl. At ATP concentrations ranging from 0.1 to 3.0 mM, the presence of 20 mM KCl leads to a 10-fold increase in the enzyme's affinity for ATP. ATPase activity is greatest at a pH of approx. 8. In addition to ATP, GTP serves as a substrate, but CTP, ADP, AMP and *p*-nitrophenyl phosphate do not. Either Mg^{2+} or Mn^{2+} is required for activity and cannot be replaced by Ca^{2+} or Zn^{2+}. The ATPase activity of goblet cell apical membranes is inhibited by neither the typical ($Na^+ + K^+$)-ATPase inhibitors, ouabain and orthovanadate, nor by the typical mitochondrial F_1F_0-ATPase inhibitors, azide and oligomycin. Although 1.5 μM DCCD is ineffective, 150 μM DCCD leads to total inhibition of ATPase activity. The ATPase activity of goblet cell apical membranes is stimulated not only by K^+, but also, in order of decreasing effectiveness, by Rb^+, Li^+, Na^+ and even Mg^{2+}. Replacement of Cl^- by Br^-, F^- and HCO_3^- has less influence than variation of the cations. However, replacement of Cl^- by NO_3^- inhibits strongly this ATPase activity. The ATPase activity described above is characteristic of the alkali metal ion pump containing apical membranes of goblet cells and is not enhanced to a similar degree in other purified midgut epithelial cell plasma membrane segments. Its localization, its broad cation specificity and its insensitivity to ouabain all mimic properties of active ion transport by the lepidopteran midgut and suggest this ATPase as a possible key component of the lepidopteran electrogenic alkali metal ion pump.

Introduction

Gastrointestinal and sensory epithelia of insects contain a unique, electrogenic, alkali metal ion pump. In gastrointestinal epithelia, such as salivary glands, midgut, Malpighian tubules, and perhaps, rectum, the pump drives fluid secretion, regulates ionic composition, and may aid nutrient absorption [1]. In sensory epithelia, such as cuticular sensilla, it is the main energy source for the generation of receptor currents [2]. In both gastrointestinal and sensory epithelia, the pump generates a lumen-positive voltage in excess of 100 mV and is suggested to reside in 8–10-nm particles, portasomes, studding the cytoplasmic side of the apical plasma membrane [2,3]. Since potassium is the ion usually pumped in both types of epithelia, the alkali metal ion pump has often been referred to

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Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Mops, 4-morpholinopropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

simply as the potassium pump.

Primary active ion transport by most animal epithelia utilizes a type of pump generally called the sodium pump [4]. The insect alkali metal ion pump is a well-documented exception and appears to be unique in several ways: unlike the Na^+-K^+ exchange pump, which is but partially electrogenic, pumps K^+ into cells, is inhibited by ouabain, and resides in basolateral membranes of epithelial cells, the insect alkali metal ion pump is fully electrogenic [5], is not inhibited by ouabain [6], pumps K^+ out of cells, and resides in the apical membranes of epithelial cells [2,7]. The insect alkali metal ion pump resembles the F_1-F_0-ATPase of phosphorylating membranes in its location in membranes studded with 8–10-nm particles and its electrogenicity, but differs in its apparent preference for alkali metal ions.

The alkali metal ion pump is quickly inhibited by oxygen lack [8,9] and its activity is directly correlated with the cytoplasmic ATP pool [10]. Therefore, it is highly reasonable that the molecular basis of the pump lies in an alkali metal ion-stimulated ATPase which couples ATP hydrolysis directly to the electrogenic movement of ions. Such an ATPase has been identified in crude membrane extracts from the midgut of the lepidopteran larva, *Manduca sexta* [11–13] and from the labellum of the dipteran adult, *Protophormia terraenovae* [14,15]. Methods have been developed for the isolation of plasma membrane fragments from four different regions of *M. sexta* midgut epithelial cells, including especially the alkali metal ion pump containing goblet cell apical membranes [16].

In this paper, we demonstrate a cation-stimulated ATPase activity localized in purified goblet cell apical membranes, describe its characteristics, and discuss the evidence that it is a candidate for the alkali metal ion pump.

Materials and Methods

Experimental insects. Fifth instar larvae of *M. sexta* (Lepidoptera, Sphingidae), weighing 4.5–6 g, were used for all experiments. They were purchased as second or third instar larvae from Carolina Biological Supply Co. and reared at 26°C on the artificial diet supplied with the larvae.

Isolated membrane preparations. For crude extract preparations, the posterior portion of one larval midgut was removed and cut into small squares of tissue as described by Cioffi and Wolfersberger [16]. The tissue pieces were suspended in 0.3 ml of 0.26 M sucrose/5 mM EDTA/5 mM Tris, buffered to pH 8.1 (buffer A) and homogenized for 3 min in an ice-cold Duall 20 homogenizer. The homogenate was diluted with 4 ml of buffer A and centrifuged at $100\,000 \times g$ for 60 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5 ml buffer A containing 0.6% bovine serum albumin.

Goblet cell apical membranes, columnar cell apical membranes, basal membranes and lateral membranes were isolated by the procedures of Cioffi and Wolfersberger [16]. The isolation of goblet cell apical membranes and lateral membranes required an overnight centrifugation, whereas the preparation of crude extract pellets, columnar cell apical membranes and basal membranes required less than 1 day. Therefore, the crude extract pellet, columnar cell apical membranes and basal membranes were stored overnight at 4°C as suspensions in buffer A containing at least a 10-fold higher concentration of bovine serum albumin than insect protein; the high concentration of bovine serum albumin was used to minimize the effects of any endogenous proteinase activity on the membrane preparations.

To facilitate the access of substrates and effectors [1], each enzyme preparation was shocked osmotically [17–19] by resuspending it in 5 mM Tris-Mops/0.32 mM EGTA (pH 7.5). Immediately before use, all enzyme suspensions were centrifuged at $100\,000 \times g$ for 60 min at 4°C. The resulting pellets were resuspended in 5 mM Tris-Mops/3.2 mM mercaptoethanol/0.32 mM EGTA/0.25% ethanol/0.1% bovine serum albumin (pH 7.5) buffer.

ATPase and phosphatase assays. Standard assays consisted of either 1–6 μg protein/ml goblet cell apical membrane or 10–100 μg protein/ml other enzyme preparations, 0.3 mg/ml bovine serum albumin, 50 mM Tris-Mops, 1 mM mercaptoethanol, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.08% ethanol, 0.5 mM sodium azide, 1 mM Tris-ATP, and either 0 or 20 mM KCl. Except in the pH optimum experiments, the pH was adjusted to

8.0 with Tris base. All assay mixtures had a final volume of 160 μl.

The mixtures were preincubated in the absence of the substrate for 20–30 min at 30°C, and assays were started by addition of the substrate. Preincubation times up to 60 min had no influence on the enzyme activity of standard assays. The usual incubation time was 12 min; the standard incubation temperature was 30°C. All samples were run in triplicate. Reactions with all substrates, except *p*-nitrophenyl phosphate, were stopped by placing the assay tubes in liquid nitrogen. Reactions with *p*-nitrophenyl phosphate as the substrate were stopped by addition of 200 μl of 1 M NaOH. Inorganic phosphate was determined as described by Wieczorek [14]. *p*-Nitrophenol was determined at 410 nm, employing a millimolar extinction coefficient of 17.1.

Enzyme activity values with or without KCl were corrected for zero time controls. KCl-stimulated activities were calculated as the difference in substrate hydrolysis in the presence and absence of KCl. Incubations were carried out under conditions in which substrate hydrolysis was a linear function of both extract concentration and incubation time.

Cytochrome oxidase assays. Cytochrome oxidase activity was measured as the rate of oxidation of reduced cytochrome *c* according to the method of Cooperstein and Lazarow [20].

Protein determination. The protein content of pellet material was determined by the amido black method [21] modified with respect to the solubilizing mixture [14]. Thus, pellets were resuspended in a solution of 8 M urea/5% Triton X-100/0.05% Na_2CO_3, and incubated for 60 min at 22–25°C followed by 10 min at 60°C.

Chemical reagents. All reagents used were of the purest commercially available quality. ATP, GTP, CTP, ADP, *p*-nitrophenyl phosphate (all Tris salts), AMP, bovine serum albumin, ouabain, oligomycin, DCCD, sodium azide and cytochrome *c* type III were purchased from Sigma. Malachite green was purchased from Serva, Amido black from Merck and sodium orthovanadate from Fisher Scientific. The concentration of sodium orthovanadate was determined indirectly as sodium concentration by flame photometry.

Results

General features of the ATPase activity in goblet cell apical membranes

Purified goblet cell apical membranes contain an ATPase in substantial activity which is stimulated 100% by KCl (Fig. 1). KCl stimulation is half-maximal at approx. 10 mM KCl, maximal at approx. 30 mM KCl, and diminishes at higher KCl concentrations. The ATPase activity both with and without KCl is almost totally dependent upon $MgCl_2$. At present, we cannot decide whether the ATPase activity of goblet cell apical membranes reflects only one ATPase entity which is modulated by KCl, or if there are two or more ATPases one of which is activated by KCl. Therefore, we report in the following not only ATPase activities without KCl and with 20 mM KCl, but also report the difference between them – the KCl-stimulated ATPase activity.

At a concentration of 0.5 mM, the well-known mitochondrial ATPase inhibitor, sodium azide [22], inhibits the ATPase activity of goblet cell apical membranes to the same small extent at all KCl concentrations (Fig. 1). The effects of increasing azide concentrations on ATPase activity with and without 20 mM KCl are illustrated in Fig. 2. At very low concentrations of sodium azide, there is a slight decrease of ATPase activity. At sodium azide concentrations above 1 mM, the activity without added KCl is increased, while that with KCl is decreased. Therefore, the KCl-stimulated activity (difference curve) is inhibited by sodium azide at concentrations above 1 mM. Together, the results in Figs. 1 and 2 demonstrate that sodium azide at a concentration of 0.5 mM does not affect the stimulation of ATPase activity by KCl, but may prevent the expression of ATPase activity, possibly of mitochondrial origin, which is not influenced by KCl. To minimize contributions of such azide-sensitive ATPases, 0.5 mM sodium azide was routinely included in the standard assays.

The effects of pH on the azide-insensitive ATPase activity with and without KCl are shown in Fig. 3. Without KCl, the ATPase activity is only slightly influenced by changing pH. In the presence of KCl, the ATPase activity becomes strongly pH-dependent, a pH of approx. 8 being

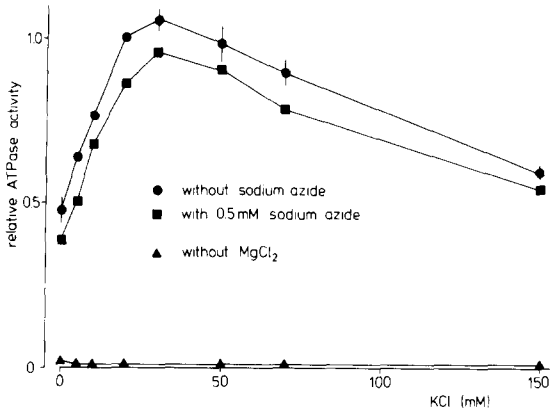

Fig. 1. ATPase activity as a function of KCl concentration. Standard assays with the concentration of KCl varied. There were three independent experiments with separate membrane preparations without sodium azide, one with 0.5 mM sodium azide, and one without $MgCl_2$. Deviations are S.E. values. The specific activity at 1.0 is 95.0 ± 5.2 μmol/mg per 60 min ($\bar{m} \pm$ S.E.).

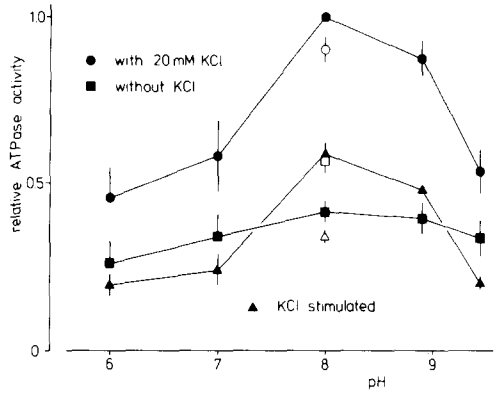

Fig. 3. ATPase activity as a function of pH. Standard assays with the pH of the buffer varied by adjustment with Tris base. Open symbols at pH 8.0: same total Tris concentration (150 mM) as at pH 9.4. There were three independent experiments with separate membrane preparations. Deviations are S.E. values. The specific activity at 1.0 is 84.8 ± 1.3 μmol/mg per 60 min ($\bar{m} \pm$ S.E.).

optimal for KCl-stimulated ATPase activity. To check if the low activity at pH 9.4 is due only to the elevated Tris concentration (150 mM) required to adjust the pH to 9.4, we tested the activity at pH 8 in the presence of 150 mM Tris (Fig. 3). Under this experimental condition, the activity without KCl is enhanced slightly, and that with KCl is inhibited slightly. Therefore, the KCl-stimulated activity at pH 8 with 150 mM Tris is only about 60% of the value obtained with 50 mM Tris. It is nonetheless nearly twice as high as the value at pH 9.4, indicating that the difference between KCl-stimulated activity at pH 8 and pH 9.4 is mainly a pH effect and only partly due to the elevated Tris concentration.

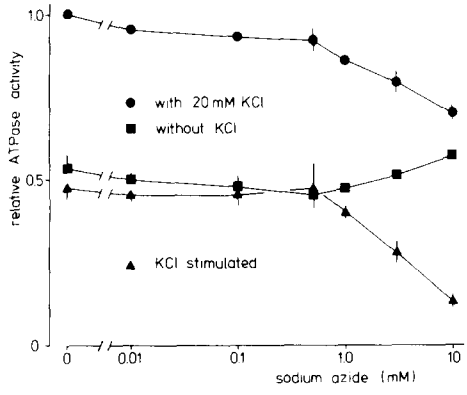

Fig. 2. ATPase activity as a function of sodium azide concentration. Standard assays with the concentration of sodium azide varied. There were three independent experiments with separate membrane preparations for each data-point, except those without sodium azide ($N = 6$) and those with 0.5 mM sodium azide ($N = 5$). Deviations are S.E. values. The specific activity at 1.0 is 92.0 ± 5.0 μmol/mg per 60 min ($\bar{m} \pm$ S.E.).

ATPase activities in other purified midgut plasma membranes

The active step in electrogenic potassium ion transport across the lepidopteran midgut epithelium takes place at the apical membrane of the goblet cells [1,7]. Therefore, it was of interest to determine whether KCl-stimulated, azide-insensitive ATPase activity at pH 8 was concentrated in several different segments of the plasma membranes of midgut epithelial cells or only the one which contains the electrogenic alkali metal ion pump.

As shown in Table I, the ATPase activity in the absence of KCl is 3–14-times greater in goblet cell apical membranes than in the other isolated membrane fractions. With KCl, it is 6–22-times greater in goblet cell apical membranes than in the other membranes. Consequently, the KCl-stimulated ATPase activity is 15–46-times greater in goblet cell apical membranes than in the other

TABLE I

ENZYME ACTIVITIES IN CRUDE EXTRACT PELLET AND IN PURIFIED PLASMA MEMBRANES OF THE MIDGUT

Standard ATPase assays with 1 mM ouabain, except for goblet cell apical membranes which were examined under standard conditions but without added ouabain. Specific ATPase activities are expressed as μmol/mg per 60 min, and specific cytochrome oxidase activity as s^{-1}/mg. The numbers of independent experiments with separate membrane preparations are given in parentheses. Mean values $\pm$ S.E. are shown. The mean amount of protein, expressed as μg per larva $\pm$ S.E., for the preparations was 3668 ± 566 in the crude extract pellet, 4.4 ± 0.2 in goblet cell apical membranes, 25.6 ± 2.7 in columnar cell apical membranes, 8.6 ± 1.7 in basal membranes and 2.6 ± 0.9 in lateral membranes. These values are similar to those reported previously [1].

Enzyme activity	Crude extract pellet (10)	Goblet cell apical membrane (6)	Columnar cell apical membrane (5)	Basal membrane (4)	Lateral membrane (4)
ATPase without KCl	3.4 ± 0.3	40.9 ± 3.0	3.0 ± 0.4	7.9 ± 1.0	12.6 ± 0.9
ATPase with 20 mM KCl	5.0 ± 0.4	87.3 ± 3.2	4.0 ± 0.7	10.9 ± 1.2	15.4 ± 1.2
KCl-stimulated ATPase	1.6 ± 0.1	46.4 ± 3.3	1.0 ± 0.2	3.0 ± 0.3	2.8 ± 0.4
Cytochrome oxidase	3.9 ± 1.0	0.8 ± 0.1	0.4 ± 0.1	1.2 ± 0.2	0.7 ± 0.1

membranes. Its activity in other plasma membrane segments is sufficiently low as to be attributable to their contamination by goblet cell apical membrane material. Clearly, the high ATPase activity is a special feature of the potassium-transporting goblet cell apical membranes.

Control experiments to check the contamination of plasma membrane fractions with inner mitochondrial membranes demonstrated a 3–10-fold reduction of specific cytochrome oxidase activity in the various purified membrane preparations as compared with the crude extract pellet (Table I). The 3–10-fold reduction in activity of the marker enzyme for mitochondrial inner membranes, cytochrome oxidase (Table I), is consistent with that reported using succinate dehydrogenase as a mitochondrial marker enzyme in similar preparations [16,23].

Further characteristics of the azide-insensitive ATPase activity in goblet cell apical membranes

ATP concentration. To determine the dependence of ATPase activity on ATP concentration at pH 8, the concentration of Mg^{2+} was adjusted to be 1 mM higher than that of ATP. Concentrations of $MgATP^{2-}$ at this pH will then deviate only slightly from the concentration of total ATP [24]. Inspection of Fig. 4 reveals that at ATP concentrations below 0.1 mM, 20 mM KCl has no detectable effect on enzyme activity. At ATP concentrations between 0.1 and 3 mM, there is an increasing amount of stimulation, and at higher ATP concentrations KCl stimulation is again decreased. The relative enhancement of activity by KCl at 17.5 mM ATP is approximately the same as at 0.1 mM ATP. If KCl-stimulated activity is treated separately, it is increased by ATP concentrations between 0.1 and 7 mM, and decreased by ATP at concentrations above 7 mM (lowermost curve of Fig. 4).

Substrate specificity. The nucleotide specificity and the divalent cation requirements of the azide-insensitive ATPase activity of goblet cell apical membranes are summarized in Table II. Without KCl, the purine nucleotides ATP and GTP are hydrolyzed at similar rates. However, the pyrimidine nucleotide, CTP, as well as ADP, AMP and *p*-nitrophenyl phosphate are much poorer substrates – as poor as is ATP in the absence of Mg^{2+}. For the KCl-stimulated activity, ATP is the best substrate, GTP is still a reasonable good substrate, but all other nucleotides, as well as *p*-nitrophenyl phosphate, are not hydrolyzed at all. Regarding the effect of divalent cations in the absence of KCl, Mn^{2+} is a much more potent activator than Mg^{2+} which in turn is much more potent than Ca^{2+}; Zn^{2+} has no influence on ATPase activity. KCl-stimulated activity is approximately the same with Mn^{2+} or Mg^{2+} but negligible with Ca^{2+} or Zn^{2+}.

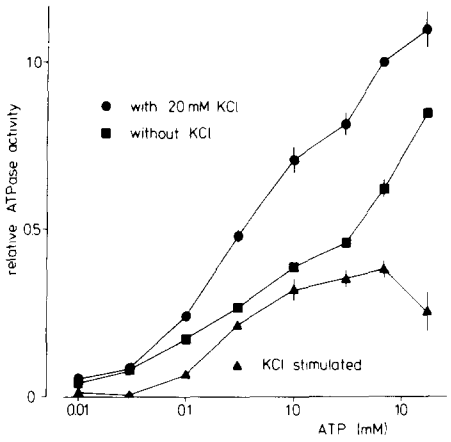

Fig. 4. ATPase activity as a function of ATP concentration. Standard assays with the concentrations of ATP and $MgCl_2$ varied. The concentrations of $MgCl_2$ are always 1 mM in excess of the ATP concentrations. There were three independent experiments with separate membrane preparations. Deviations are S.E. values. The specific activity at 1.0 is 116.7 ± 9.1 μmol/mg per 60 min ($\bar{m} \pm$S.E.).

Inhibitors. Potent, specific inhibitors of well-known transport ATPases have little or no effect on the ATPase activity of goblet cell apical membranes (Table III). Thus, inhibitors of mitochondrial ATPases such as oligomycin, 1 mM azide and 1.5 μM DCCD [22] are ineffectual (see also Fig. 2). Ouabain, the typical inhibitor of the ($Na^+ + K^+$)-ATPase [25], is likewise ineffectual. Orthovanadate, an inhibitor of those ATPases which form stable phosphoenzyme intermediates [26], is virtually ineffectual. Fluoride, an inhibitor of the gastric ($H^+ + K^+$)-ATPase [27], is ineffectual, even at a concentration of 30 mM (Table IV). Two agents were found to inhibit the ATPase activity of goblet cell apical membranes. DCCD at a concentration of 150 μM inhibits activity by approx. 90% both with and without KCl (Table III). Nitrate at a concentration of 30 mM inhibits activity without KCl by more than 50% (Table IV).

Ion activation. Since the alkali metal ion pump in lepidopteran midgut has a rather broad cation specificity [28], it was important to examine the effects of salts other than KCl on the ATPase activity of goblet cell apical membranes. We replaced 30 mM K^+ by a series of monovalent and divalent cations, and replaced 30 mM Cl^- by various monovalent anions (Table IV). Replacement of K^+ by NH_4^+ has no effect, but replacement of K^+ by Rb^+, Li^+ and Na^+ has significant effects. All of these monovalent cations stimulate the ATPase activity of goblet cell apical mem-

TABLE II

ENZYME ACTIVITY AS A FUNCTION OF THE SUBSTRATE

Standard assays with the indicated organic phosphates and divalent salts at 1 mM each. Specific activity (expressed in μmol/mg per 60 min; $\bar{m} \pm$S.E.) for the 100% value without KCl is 40.2 ± 3.2, with 20 mM KCl is 82.1 ± 6.5, and for KCl stimulation is 41.9 ± 3.7. Each value is from three independent experiments with separate membrane preparations, except for the 100% values for which there were nine experiments. Mean values $\pm$ s.E. are shown. NPP, *p*-nitrophenyl phosphate.

Substrate	Relative specific activity		
	without KCl	with 20 mM KCl	KCl-stimulated
ATP + $MgCl_2$	100	100	100
GTP + $MgCl_2$	112 ± 1	85 ± 7	61 ± 10
CTP + $MgCl_2$	20 ± 3	6 ± 1	0 ± 0
ADP + $MgCl_2$	12 ± 2	6 ± 1	1 ± 1
AMP + $MgCl_2$	13 ± 3	6 ± 2	1 ± 1
NPP + $MgCl_2$	13 ± 1	6 ± 1	0 ± 0
ATP + none	10 ± 3	5 ± 1	1 ± 1
ATP + $MnCl_2$	182 ± 4	143 ± 3	107 ± 4
ATP + $CaCl_2$	46 ± 2	26 ± 1	7 ± 1
ATP + $ZnCl_2$	14 ± 1	8 ± 1	3 ± 2

TABLE III

EFFECT OF INHIBITORS ON ATPase ACTIVITY

Standard assays with the inclusion of inhibitors at the concentrations indicated. Specific activity (expressed in μmol/mg per 60 min; $\bar{m} \pm$S.E.) for the 100% value without KCl is 37.4 ± 5.3, with 20 mM KCl is 76.8 ± 10.6, and for KCl stimulation is 39.4 ± 5.9. Each value is from three independent experiments with separate membrane preparations, except for the 100% values for which there were five experiments. Mean values $\pm$ S.E. are shown.

Inhibitor	Relative specific activity		
	without KCl	with 20 mM KCl	KCl-stimulated
None	100	100	100
1 mM ouabain	95 ± 3	99 ± 1	102 ± 3
20 μg/ml oligomycin	90 ± 2	87 ± 5	85 ± 7
1 μM Na_3VO_4	99 ± 2	101 ± 2	103 ± 3
100 μM Na_3VO_4	80 ± 3	85 ± 2	91 ± 5
1.5 μM DCCD	92 ± 3	94 ± 1	95 ± 3
150 μM DCCD	14 ± 3	6 ± 0	1 ± 0

TABLE IV

INFLUENCE OF SALTS ON ATPase ACTIVITY

Standard assays with the inclusion of salts at the indicated concentrations. The specific activity for the 100% value is 79.7 ± 6.6 μmol/mg per 60 min ($\bar{m}$ ± S.E.). The numbers of independent experiments with separate membrane preparations are given in parentheses. Mean values ± S.E.M. are shown.

Salt	Relative specific activity
No addition	44 ± 2 (6)
30 mM KCl	100 (6)
30 mM RbCl	107 ± 3 (3)
30 mM NH_4Cl	99 ± 1 (3)
30 mM LiCl	86 ± 2 (4)
30 mM NaCl	81 ± 4 (6)
30 mM NaCl + 30 mM KCl	88 ± 2 (3)
15 mM NaCl + 15 mM KCl	98 ± 2 (3)
30 mM $MgCl_2$	56 ± 2 (3)
30 mM $CaCl_2$	36 ± 4 (3)
15 mM $MgCl_2$	79 ± 3 (4)
15 mM $CaCl_2$	49 ± 3 (4)
30 mM KBr	91 ± 1 (3)
30 mM KF	103 ± 4 (3)
30 mM $KHCO_3$	98 ± 3 (3)
30 mM KNO_3	21 ± 1 (3)

branes; the 'best' cation, Rb^+, induces a 32% higher activity than the 'worst' cation, Na^+. Mixtures of KCl and NaCl do not produce any synergistic effect, as would be expected for an ($Na^+ + K^+$)-ATPase [29]. Regarding divalent cations, 30 mM Mg^{2+} stimulates only slightly and 30 mM Ca^{2+} inhibits the ATPase activity. Lowering the divalent cation concentration to 15 mM (the Cl^- now being 30 mM) leads to a stimulation by Mg^{2+} which is similar to that of Na^+, whereas Ca^{2+} has almost no effect. The replacement of Cl^- by Br^-, F^- and HCO_3^- leads to smaller differences than those obtained by the variation of cations. The 'best' anion, F^-, produces a 13% higher activity than the 'worst' anion, Br^-. As was already mentioned above, NO_3^- is a strong inhibitor of the ATPase activity. HCO_3^-, which is a potent activator of the mitochondrial F_1F_0-ATPase [22], has the same effect as the tested halogen ions. In summary, the activity of the ATPase in goblet cell apical membranes is more influenced by variation of cations than anions, although anion variation is not without influence.

Discussion

KCl-stimulated ATPase activity in crude membrane preparations

The properties of the ATPase activity of purified goblet cell apical membranes resemble those reported for plasma membrane ATPase activities in partly purified preparations from *M. sexta* larval midgut [11–13]. Some differences between the present results and those of Deaton [13] may originate from his use of NO_3^- as a counterion for Mg^{2+}. NO_3^- has been shown in this study to be a strong inhibitor of this ATPase activity. The properties of the ATPase activity of goblet cell apical membranes are also very similar to those of ATPase activities in the labellum of the blowfly which have been proposed as candidates for the electrogenic K^+ pump in insect cuticular sensilla [14,15].

Localization of ATPase activity in goblet cell apical membranes

The substantial activity of the KCl-stimulated ATPase in goblet cell apical membranes results from a 20-fold purification with respect to the pellet of the crude extract. In the other purified plasma membrane segments (as is in the crude extract), specific KCl-stimulated ATPase activity is barely detectable. In the absence of KCl, goblet cell apical membranes still possess the highest specific ATPase activity, but the purification factor is only 12. Relative to the crude extract, the specific ATPase activities are more or less unchanged in columnar cell apical membranes, but increased 2–4-fold in basal and lateral membranes (Table I).

As a consequence of the high purification ratios for the ATPase activity in goblet cell apical membranes, we would expect the activities in at least some of the other plasma membrane segments to be reduced. That this is not the case may be due to the loss of intracellular membranes and of muscle components which are present in the crude extract. Furthermore, it is likely that the activity in basal and lateral membranes is due to goblet cell apical membrane contamination; it is relatively easy to prepare columnar cell apical membranes uncontaminated by goblet cell apical membranes but difficult to prepare uncontaminated basal

membranes and extremely difficult to prepare uncontaminated lateral membranes [16]. Even taking into account that goblet cell apical membrane is only a small part of total midgut cell plasma membranes, the amount of protein in the final fraction of goblet cell apical membranes is small. This indicates that the high purification by the separation procedure of Cioffi and Wolfersberger [16] is obtained at the expense of a considerable loss of membrane material, as is often found with highly purified membrane preparations [30,31].

Alkali metal ion stimulation of goblet cell apical membrane ATPase activity

The substantial ATPase activity without KCl together with the large KCl stimulation pose two crucial questions. (1) Is there only one ATPase which is stimulated by KCl or are there two or more ATPases at least one of which is KCl-sensitive while the rest are KCl-insensitive? (2) Is the ATPase activity specifically stimulated by ions or should the influence of ions better be interpreted as an unspecific salt effect?

One or more ATPases. Goblet cell apical membrane ATPase activity is enhanced 2-fold by the presence of 20–50 mM KCl. About 12% of the ATPase activity without KCl (and about 6% of that with KCl) may be due to unspecific phosphatase activities, as revealed by the substrate specificities: the hydrolysis rates for CTP, ADP, AMP and *p*-nitrophenyl phosphate are nearly identical and are similar to that of ATP when Mg^{2+} is absent or replaced by Zn^{2+} (Table II). Moreover, orthovanadate, a potent inhibitor of phosphatases [32], affects to a small but significant extent the ATPase activity (Table III). If the alleged phosphatase activities are subtracted from total ATPase activities, then both the ATPase activity without KCl and that with KCl exhibit similar properties: GTP is hydrolyzed at a rate comparable to ATP (114 or 84%), CTP, ADP, AMP and *p*-nitrophenyl phosphate are poor substrates at best, Mn^{2+} stimulates more than Mg^{2+} (193 or 146%), Ca^{2+} is much less effective than Mg^{2+} (39 or 21%), and Zn^{2+} is nearly ineffective (2% in either case). Furthermore, the inhibitor specificities of both enzyme activities are similar (Table III). Consequently, there is reason to believe that there is only one ATPase entity in the alkali metal ion-transporting goblet cell apical membranes, the activity of which is stimulated by KCl. Nevertheless, at present we cannot reject the alternative hypothesis that two or more ATPases with similar properties are present, at least one of which, like the $(Na^+ + K^+)$-ATPase or the gastric $(H^+ + K^+)$-ATPase [25,33] is cation-activated.

Specific ion stimulation or unspecific salt effect. The isolated lepidopteran midgut transports alkali metal ions rather unspecifically [34]. Therefore, one might expect that an ATPase involved in this alkali metal ion transport system would also show a rather broad cation selectivity. The cation sensitivity of the ATPase activity of goblet cell apical membranes (Table IV) is in accordance with this expectation. Nevertheless, broad salt selectivity is also a property of a variety of proton-pumping ATPases the maximal velocity of which is increased by salts in a more or less unspecific manner [35–39]. By contrast, the effect of KCl on the ATPase activity of goblet cell apical membranes is not unspecific, even if the activity is treated as a single enzyme: the sensitivity for ATP is enhanced 10-fold by KCl, in a concentration range from 0.1 to 3 mM ATP (Fig. 4). Fig. 5 shows that KCl exerts its influence on the affinity and not on the maximal velocity: the KCl stimulation, expressed as the ratio of activities with and without KCl, increases from a value near 1 at 30 μM ATP to a maximum value near 1.8 at approx. 1 mM, and decreases symmetrically with higher ATP concentrations.

Unique properties of the ATPase activity in goblet cell apical membranes

The ATPase activity of goblet cell apical membranes displays characteristic substrate preferences, salt selectivities and inhibitor sensitivities which distinguish it from several well-known ATPases. It is not homologous to the gastric $(H^+ + K^+)$-ATPase, as suggested by several authors [13,33,40]. In contrast to the gastric $(H^+ + K^+)$-ATPase, goblet cell apical membrane ATPase activity shows a broader salt selectivity, uses GTP as substrate, cannot hydrolyze *p*-nitrophenyl phosphate, is insensitive to fluoride, and is inhibited by nitrate. Nitrate neither affects the gastric $(H^+ + K^+)$-ATPase [41] nor the plasma membrane ATPase of *Neurospora* [42], but inhibits vacuolar

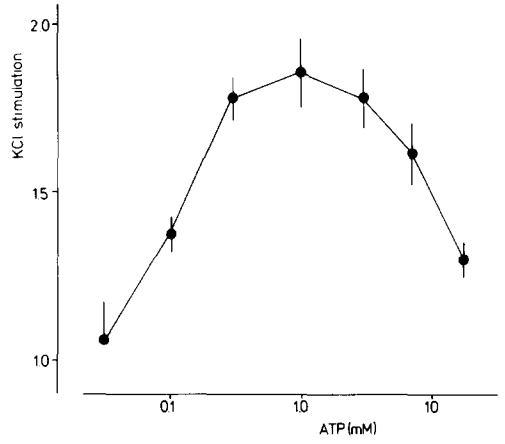

Fig. 5. KCl stimulation as a function of ATP concentration. The values for KCl stimulation are calculated from the activities shown in Fig. 4. They are expressed as the quotients obtained by the division of activities in the presence of KCl by those in the absence of KCl. Deviations are S.E. values.

ATPases of eukaryotic microorganisms and of plants [42–44]. More importantly, the ATPase activity of goblet cell apical membranes is insensitive even to high concentrations of vanadate. This vanadate insensitivity distinguishes it from all $(Na^+ + K^+)$-type ATPases, including not only those found in plasma membranes of virtually all ion-transporting animal cells but also those in proton-transporting plasma membranes of fungal and plant cells (e.g., Refs. 25, 31, 33, 45 and 46).

The ATPase activity of goblet cell apical membranes does not seem to belong to the same class as the mitochondrial F_1F_0-ATPase [22] as may be concluded from its insensitivity to oligomycin, or to azide concentrations up to 1 mM, or to low DCCD concentrations. In its sensitivity to DCCD at concentrations higher than those required for inhibition of the mitochondrial F_1F_0 enzyme, the ATPase activity of goblet cell apical membranes resembles the ATPases in various acidic organelles [47–51].

Although it cannot be excluded, there is presently no evidence that the ATPase activity of goblet cell apical membranes could be an ecto-ATPase, such as has been shown to exist in a variety of plasma membranes [52–55]: ecto-ATPases frequently display a broader substrate specificity, use Ca^{2+} as well as Mg^{2+} as cofactor, are not sensitive to nitrate, and do not seem to be influenced in their substrate affinity by KCl.

Relation of ATPase activity to alkali metal ion pump

We have demonstrated in this paper several characteristics of an ATPase activity in goblet cell apical membranes. Although we did not study transport characteristics simultaneously, the well-known physiology of midgut transport [3,12] as well as the proof that goblet cell apical membranes perform the electrogenic transport of alkali metal ions [1,7] provide sufficient reason to discuss the evidence that the ATPase activity in goblet cell apical membranes is a candidate for the electrogenic alkali metal ion pump.

First of all, as mentioned above, the ATPase activity is concentrated in the same midgut cell plasma membrane segment which has been shown to perform the electrogenic alkali metal ion transport [1,7]. Its activity is stimulated by KCl; its sensitivity for ATP is greatly enhanced by the presence of KCl. Goblet cell apical membrane ATPase activity and midgut cation translocation have several additional strikingly similar characteristics. The pH optimum for alkali metal ion transport by the isolated midgut, 8.3 [56], is essentially the same as the pH optimum for ATP hydrolysis (Fig. 3). Electrogenic alkali metal ion transport, like the ATPase activity, is not inhibited by ouabain [6,57]. Half-maximal stimulation of both ATPase activity (Fig. 1) and alkali metal ion transport [28] is obtained at a K^+ concentration of approx. 10 mM. The isolated midgut pumps K^+ about as well as Rb^+, whereas Li^+ and Na^+ are transported at significantly lower rates [34]. The ATPase activity of goblet cell apical membranes is stimulated about equally by K^+ and Rb^+, but less by Li^+ and Na^+.

Unlike alkali metal ion transport, the ATPase activity of goblet cell apical membranes can be stimulated by Mg^{2+} (Table IV). However, the differing effects of Mg^{2+} are understandable: Mg^{2+} stimulation of alkali metal ion transport by the intact midgut would be masked by active Mg^{2+} transport in the opposite direction [58].

Whether or not the ATPase activity of midgut goblet cell apical membranes is a key component of the insect electrogenic alkali metal ion pump

will not be decided until this activity has been purified and the transport system has been reconstituted. However, the circumstantial evidence discussed above is sufficient to cause us to pursue these goals vigorously.

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