BBA 75 152

AN ION TRANSLOCASE SYSTEM FROM RABBIT INTESTINAL MUCOSA. PREPARATION AND PROPERTIES OF THE (Na^+-K^+)-ACTIVATED ATPase

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(Received January 12th, 1968)

SUMMARY

1. A particulate enzyme complex from rabbit intestinal mucosa exhibiting ($Na^+ + K^+$)-stimulated ATPase activity has been isolated and partially purified.

2. The ATPase activity exhibits an asymmetric stimulatory response to Na^+ and K^+ and is inhibited by ouabain and Ca^{2+}.

3. Specific inhibition of the ATPase activity by mersalyl, phenolphthalein, and culture filtrates from *Vibrio cholerae* are also reported.

4. It is concluded that the ATPase activity is a moiety of the membrane-associated ion translocase system of rabbit intestinal mucosa and that mucinase activity in the culture filtrates selectively destroys or inactivates this system *in vitro*.

INTRODUCTION

In the course of our *in vitro* studies on the biochemistry of *Vibrio cholerae* virulence we found it useful, in investigating the active sodium pump inhibitor reportedly elaborated *in vitro* and *in vivo*[1] by choleragenic strains of *V. cholerae*, to have available an enzymic equivalent of the membrane-associated ion translocase system of intestinal mucosa. Therefore, we have isolated and partially purified from rabbit intestine, a particulate ion-activated ATPase complex which also displays K^+-stimulated neutral phosphatase activity[2,3].

It is the purpose of this report to describe the general properties of the ATPase and to present data which show that *V. cholerae* culture filtrates inhibit the ion translocase system of rabbit intestinal epithelium *in vitro*.

METHODS AND MATERIALS

Preparation of the ion translocase. The isolation technique employed was a modification of the procedure developed by AHMED AND JUDAH[4] for the isolation of ion-stimulated ATPases from mammalian tissues. Adult rabbits of either sex were sacrificed by an air embolism introduced through the marginal ear vain. The upper $^1/_3$ to $^1/_2$ of the small intestine was rapidly removed and rinsed thoroughly inside and out with cold running tap water. 5 to 10-cm lengths of gut were then washed with resuspension medium[4] and the mucosa was expressed from each segment onto an ice-

cold glass plate by gently stroking the outside of the flattened section with the edge of a microscope slide. The cell suspension was diluted with a 10× volume of homogenizing medium[4] and homogenized with 25 complete strokes of a tight-fitting pestle in a glass–teflon homogenizer. Further fractionation of the homogenate by the AHMED AND JUDAH procedure yielded a preparation equivalent to their R_1 fraction. This preparation has been found to be stable at —50° for at least 6 months but loses approx. 50 % of its remaining specific activity as a consequence of the initial and each subsequent freeze–thaw cycle.

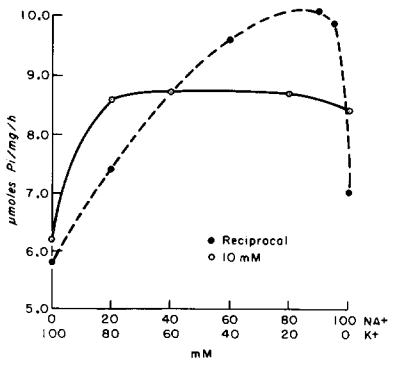
Assay techniques. 10 to 100 μg of enzyme were preincubated in a total volume of 1.0 ml ATPase medium[4] at 38° for 5 min. The reaction was initiated with ATP and terminated after 10 to 30 min with 0.5 ml of cold 9 % perchloric acid. Experimental tubes were routinely run in triplicate with a corresponding blank (ATP added after $HClO_4$) for each set. Liberated phosphate was determined as in ref. 4.

Protein was estimated by the technique of GORNALL, BARDAWILL AND DAVID[5]. Filtrates from peptone-grown cultures of *V. cholerae* were prepared as previously described[2,3]. All chemicals were of the best analytical grades commercially available.

RESULTS

Properties of the mucosal ATPase. It is now well established that there are several unique criteria for membrane-bound ATPases that must be fulfilled before any particular ATPase activity can be considered to be part of an ion translocase system[6–8]. The essential characteristics include: a reciprocal requirement for Na^+ and K^+ for maximum activity; specific inhibition by ouabain; inhibition by Ca^{2+}; and a pH optimum between 7.0 and 8.0. In order to determine whether the intestinal ATPase activity met the basic requirements it was tested within these accepted parameters.

Fig. 1 shows data obtained when mucosal ATPase activity was measured as a function of the ratio of Na^+ to K^+ in the incubation medium. The optimum ratio for



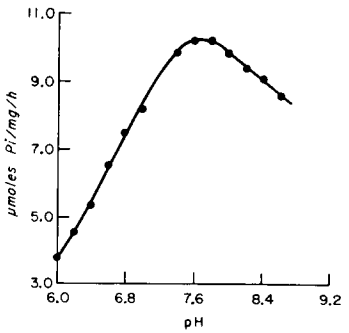


Fig. 1. ATPase activity as a function of Na^+ and K^+ concentration. The assays were run as described in the METHODS section. The open circles represent values obtained when Na^+ was varied from 0 to 300 mM (not shown) at a constant 10 mM K^+. Closed circles show the values observed when the Na^+/K^+ ratios are those shown on the abscissa.

Fig. 2. ATPase activity as a function of pH. The assays were run as described in the METHODS section. The buffer system from pH 6.0 to 7.2 was imidazole–HCl; above pH 7.2, Tris–HCl.

activity appeared to obtain at 10 mM K^+ and 90 mM Na^+. The asymmetric shape of the curve suggested that K^+ was stimulatory at low concentrations (5 to 10 mM) and inhibitory at levels above 10 mM. When varying levels of Na^+ were added to a constant low level (10 mM) of K^+ and ATP hydrolysis was followed, ATPase activity was not appreciably affected over a range of 20 to 100 mM Na^+ showing that the major effect is due to K^+. This asymmetry with regard to relative affinity for Na^+ and K^+ is characteristic of ion translocase systems.

ATP hydrolysis by the mucosal enzyme as a function of pH is recorded in Fig. 2. The rather sharp pH optimum for the reaction was found to be pH 7.6 with a more pronounced drop in activity on the acid than on the alkaline side. The decreased slope of the curve in the alkaline range may reflect a broader tolerance of the enzyme towards high pH since the cells of the upper small intestine (the source of the complex) are normally exposed to an alkaline environment.

In a series of experiments employing 16 preparations with specific activities ranging from 5.5 to 36.0 the average inhibition by ouabain was 50 % (range 38 to 61 %). Sodium stimulation of the ATPase paralleled ouabain inhibition at all levels of specific activity tested.

It has been demonstrated by a variety of techniques that Ca^{2+} (ref. 8), mercury diuretics such as mersalyl[9], and cathartics like phenolphthalein[10], markedly reduce active sodium transport *in vivo* and *in vitro*. Table I contains data summarizing the effects of these agents on mucosal ATPase activity. Mersalyl was found to be more effective in inhibiting mucosal ATPase than ouabain. As was the case with ouabain,

TABLE I

INFLUENCE OF INHIBITORS OF ACTIVE SODIUM TRANSPORT ON INTESTINAL EPITHELIUM ATPase ACTIVITY

The complete medium contained in a total volume of 1.0 ml: 2 mM $MgSO_4$, 30 mM Tris–HCl (pH 7.6), 10 mM KCl, 100 mM NaCl, 2 mM Tris–ATP. Incubation was for 20 min at 38°. Liberated phosphate was determined as described in METHODS. Each value is an average of triplicate determinations. The control in experiment 4 contained an amount of ethanol equal to that in which the phenolphthalein was dissolved. 10 % ethanol alone decreases ATPase specific activity approx. 5 % under these assay conditions.

Expt.	*μmoles phosphate* $mg^{-1}\ h^{-1}$	% *inhibition*
1. Complete	33.0	0
+ 10^{-3} M ouabain	12.9	61
+ 10^{-4} M mersalyl	6.9	79
2. Complete	9.6	0
− sodium	5.2	46
− sodium + 10^{-4}M mersalyl	3.9	59
3. Complete	8.5	0
+ 5 mM Ca^{2+}	4.4	48
+ 5 mM Ca^{2+} + 1 mM ouabain	3.9	54
4. Complete	7.1	0
+ 10^{-4} M phenolphthalein	3.6	49
+ 10^{-5} M phenolphthalein	5.4	24
+ 10^{-7} M phenolphthalein	6.2	13

maximal mersalyl inhibition required sodium ions and was only slightly inhibitory in media lacking sodium (Expt. 2, Table I) indicating that this agent has little or no effect on the Mg^{2+}-stimulated component of the ATPase activity at the concentrations routinely employed in our assays.

Of particular interest to us was the observation that the mucosal ATPase was sensitive to low levels of phenolphthalein. The concentrations employed in this experiment were of the same order of magnitude as those previously shown to inhibit active sodium transport in everted sacs of rabbit intestine[10].

Effects of V. cholerae filtrates on mucosal ATPase activity. Our primary purpose in isolating and characterizing this enzyme complex was to provide a system which would allow us to test the effects of *V. cholerae* culture filtrates on the active sodium transport system of rabbit intestine *in vitro*. Table II contains data from a typical experiment in which a sterile filtrate from a choleragenic strain of *V. cholerae* (VC 12, Ogawa) was added to the standard ATPase mixture. In this experiment the unheated filtrate inhibited ATPase activity to approx. 75 % of the value obtained with 10^{-3} M ouabain and 65 % of that of 10^{-4} M mersalyl. In other experiments titration of the ATPase with increasing amounts of filtrate yielded a curve with a maximum inhibitory level equivalent to the maximal ouabain inhibition of the particular ATPase preparation being tested[11].

TABLE II

EFFECT OF *V. cholerae* CULTURE FILTRATE ON RABBIT INTESTINAL MUCOSA ATPase ACTIVITY

Assay conditions as in Table I. Test materials were added in 0.05-ml amounts and preincubated 5 min at 38° before addition of ATP to the assay mixtures.

Conditions	*μmoles phosphate mg^{-1} h^{-1}*	*% inhibition*
Control	33	0
+ filtrate	17.7	46
+ boiled filtrate	34.3	0
+ uninoculated media	34.0	0
+ 10^{-3} M ouabain	12.9	61
+ 10^{-4} M mersalyl	6.9	79

Boiled filtrates or uninoculated media had no effect on ATPase activity. Preincubation of the enzyme and filtrates for a period of at least 5 min prior to ATP addition was found necessary to achieve maximum inhibition. Adding ATP before or concomitant with filtrate addition resulted in loss of all inhibition. Simultaneous addition of ouabain and filtrate caused no more inhibition than either substance alone showing that their effects are not additive[2,3,11].

DISCUSSION

The alkali ion response and the pH effects summarized in Figs. 1 and 2 are typical of many ion translocase systems already described[6-8]. These data along with the ouabain sensitivity of the preparation constitute sufficient evidence for the

conclusion that the modified isolation procedure described here yields enzyme complexes approx. 50 % pure in terms of their ion translocase component. Application of the same procedure to rabbit kidney cortex and dog intestinal mucosa has resulted in enzymes 2 to 3 times as active which are up to 90 % ouabain inhibitable.

Since our main concern was the total amount of ouabain-sensitive (Na^+)-activated ATPase present in each preparation we employed 10^{-3} M ouabain routinely. This concentration is at least a 10-fold excess over what is actually required for complete inhibition. The calcium inhibition observed is most likely due to a competition between Mg^{2+} and Ca^{2+} for ATP which rather tenaciously binds both ions.

The use of mersalyl inhibition as a criterion of active sodium transport is certainly not as well established as Na^+ activation or ouabain sensitivity even though there is reason to believe that its diuretic action *in vivo* may be due to a specific interference with kidney sodium pump mechanisms[9,13]. Mersalyl inhibition in our system was used simply as an added measure of the inhibitable portion of the complex. Unlike ouabain, which reaches an inhibition maximum, mersalyl at 10^{-3} M results in 50 % inhibition of the Mg^{2+}-stimulated ATPase and thus appears to cause inhibition by at least two different mechanisms affecting each moiety separately.

PHILLIPS *et al.*[10] recently showed that 10^{-3} M phenolphthalein inhibited active ion flux approx. 40 % in rabbit ileal loops *in vivo* and that sodium transport was completely cut off in short-circuited frog skin preparations at this inhibitor concentration. This led them to suggest that the diarrheal effect of phenolphthalein and several other commonly used cathartics might be due to specific inhibition of the intestinal active sodium pump mechanism. Our data with phenolphthalein are consistent with their results in that 10^{-5} M phenolphthalein is approx. 50 % inhibitory for the ouabain-sensitive moiety of the complex while 10^{-3} M caused complete inhibition of the ion-stimulated ATPase activity.

The effects of the *V. cholerae* filtrates on the ATPase were originally thought to be due to a specific inhibitor of the type described by PHILLIPS[1]. Specificity for the ion-activated portion of the enzyme complex was indicated by the observation that ouabain *plus* filtrate was no more inhibitory than either one alone. However, the further discovery that the inhibitor was heat labile and required preincubation with the enzyme in the absence of ATP to produce its effect suggested that the inhibitor might be an enzyme[2,3]. Extensive investigation has shown that the inhibitor is identical with Vibrio mucinase and that its production by *V. cholerae* can be induced by growing the organism in culture media containing low concentrations of the enzyme described in this communication[11].

The basic significance of these findings is that a degradative Vibrio enzyme which is inducible by specific components (the ATPase complex) of rabbit intestine can be shown to selectively inhibit an essential function (ion translocation) of the isolated complex. The mechanism of mucinase inhibition of the ATPase and its associated K^+-stimulated neutral phosphatase activity will be described elsewhere. The selectivity of the mucinase for the ion-stimulated moiety of the complex may offer some advantage in structural studies on the translocase especially since it appears that substrate (ATP) protects the ATPase against the action of the mucinase[11].

Our conclusion, based on the data presented above is that the ATPase activity described here is part of the ion translocase complex of the intestinal epithelium of the rabbit and that at this level of purification (approx. 50 %) can be employed as a useful

in vitro analytical tool in the study of the biochemical mechanisms of *V. cholerae* virulence.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mrs. MARY T. LANKFORD is gratefully acknowledged. This work was supported by N.I.H. grants AI-06219 and AI-07772 and by a Public Health Service Research Career Development Award (K3-AI-9018) to S.H.R.

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