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INFLUENCES OF CATIONIC BACTERICIDAL AGENTS ON MEMBRANE ATPase OF BACILLUS SUBTILIS*

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SUMMARY

The cationic bactericidal agents (β-lysin, cetyltrimethylammonium bromide (CTAB), histone, and protamine sulfate) stimulated membrane ATPase (EC 3.6.1.3) of *Bacillus subtilis* ATCC 6633, while spermine, cadaverine, and putrescine-inhibited enzyme activity. Sonication of membrane ghosts caused, along with an increase in Mg^{2+}-dependent ATPase activity and a disintegration of ghost gross structure, a release of enzyme activity to the supernatant upon centrifugation at $30\,900 \times g$ for 30 min. Membrane-bound enzyme activity was not released by β-lysin or CTAB, but was released by sodium deoxycholate and by phospholipase A. CTAB stimulated ATPase activity in the absence of Mg^{2+}, whereas β-lysin, histone, and protamine sulfate did not. The CTAB-mediated stimulation and inhibition of this enzyme were reversed by phosphatidylethanolamine and phosphatidyl-L-serine when the total negative charge of added phospholipid was equal to the total positive charge of CTAB. Combinations of CTAB and phospholipid at concentrations in excess of that required to reverse the CTAB-mediated effect caused a secondary stimulation that was similarly reversed with additional phospholipid. Added phosphatidylethanolamine had very little influence on the enzyme in the absence of CTAB, while 200 μg/ml phosphatidyl-L-serine caused a modest stimulation. Sonicate ATPase was not susceptible to activation by β-lysin, histones, or CTAB. It is suggested that activation of membrane ATPase by these substances is a secondary phenomenon, dependent upon their interaction with acidic membrane compounds, e.g. phospholipids which alters the membranous environment of the enzyme.

Abbreviations: CTAB, cetyltrimethylammonium bromide; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

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INTRODUCTION

β-Lysin, a small protein about 6000 in molecular weight [1], is a thermostable component of serum and is bactericidal for a variety of gram-positive organisms. It is believed to be involved in the natural host defense mechanisms of several mammals [2, 3]. Since acidic substances such as DNA, RNA [4], and anionic phospholipids [5] inhibited its bactericidal action, β-lysin was concluded to be basic in nature. Evidence obtained from ion-exchange chromatography also points to its cationic nature [1]. It is distinct from the antibody–complement bactericidal system since it resists denaturation at 95 °C for 15 min [6] and can be separated from antibody and complement in the asbestos filtration–elution procedure [7]. The origin of β-lysin in the serum is the blood platelet [6], and it is released from platelets during the blood coagulation process [8, 9].

Data on the effects of β-lysin on cells and protoplasts of *Bacillus subtilis* indicate that the cytoplasmic membrane is the principal site for its action [10, 11]. To learn more about this interaction which leads to death of cells and lysis of protoplasts, its influence on a membrane-associated enzyme was compared with the influences of other cationic and/or bactericidal agents, e.g. histones [12], cetyltrimethylammonium bromide (CTAB) [13, 14], protamine sulfate [12], spermine [15, 16], putrescine [15], cadaverine [15], and D- and L-histidines [15], sodium deoxycholate [13] and phospholipase A.

The membrane associated adenosine triphosphatase (ATPase) of *B. subtilis* and a form of this enzyme obtained after sonication and remaining in the supernatant after centrifugation at 30 900 × *g* for 30 min (hereafter referred to as sonicate ATPase) were used for this study. The properties of the membrane-bound enzyme are described in a previous paper [17].

MATERIALS AND METHODS

Chemicals

Sources of many of the reagents have been given previously [17]. Additional materials include the following: sodium deoxycholate and CTAB, technical grade, Matheson Scientific Co.; rabbit blood serum, Pel-Freez Biologicals; lysine- and arginine-rich histones, spermine, cadaverine, putrescine, D- and L-histidines, protamine sulfate, Grade I from salmon, and *Vipera russelli* venom phospholipase A, from Sigma Chemical Co. The phospholipids were purchased from Nutritional Biochemicals Corp. Aqueous dispersions of the phospholipids were produced by vortexing.

Preparation of membrane, and enzyme and protein assays

These were as given previously [17] with the single exception that the assays in the presence of β-lysin were performed in 0.15 M Tris–HCl buffer (pH 7.8).

β-lysin preparation

The asbestos filtration–elution method of Donaldson and co-workers [7] was used to purify β-lysin from rabbit serum approx. 600-fold.

RESULTS

Stimulation of the membrane-bound Mg^{2+}-ATPase activity by cationic bactericidal agents

β-Lysin, CTAB, arginine-rich histone, and protamine sulfate were found to stimulate the membrane bound Mg^{2+}-dependent ATPase to 200, 475, 245 and 270 % of the control level, respectively, as shown in Fig. 1 a–d. The curve showing the effects of various amounts of the cationic detergent, CTAB, on enzyme activity was complex (Fig. 1b). Infrequently, a membrane preparation was obtained that was stimulated in the 20–30-μg range (see Fig. 4). Maximal stimulation occurred either at 50 or 75 μg/ml and was approx. 300 % as compared to the usual level of about 500 % (Fig. 1b and Table II). The significance of the variability is unknown. Results similar to those obtained with arginine-rich histone (Fig. 1c) were observed with a lysine-rich histone preparation. Addition of histone up to 400 μg/ml had no effect on pH.

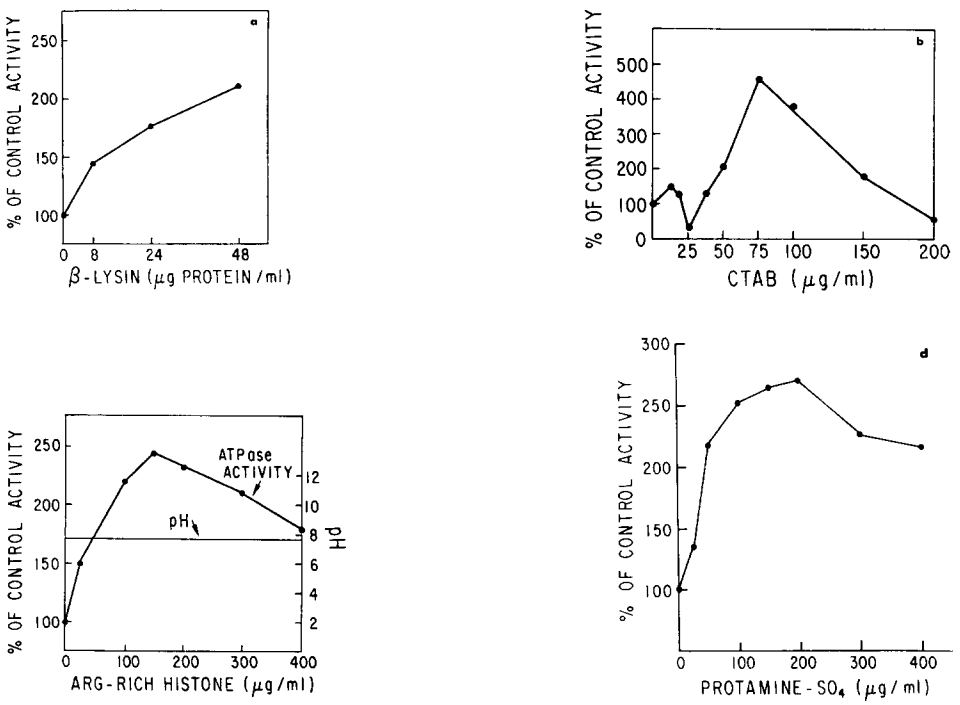

Fig. 1. Stimulation of the Mg^{2+}-membrane ATPase activity by cationic bactericidal agents. a, β-lysin; b, CTAB; c, arginine-rich histone; d, protamine sulfate. Enzyme activities in the presence and absence of β-lysin were measured in 0.15 M Tris, while those depicted in b, c, and d were measured in 0.075 M Tris. The cationic agents, CTAB, histone, and protamine sulfate were added to the assays as aqueous solutions; β-lysin was reconstituted from the lyophilized state in Tris and included in the assay as part of the buffer component.

Inhibition of the membrane Mg^{2+}-ATPase activity by diamines and a tetramine

The results shown in Fig. 2 demonstrate that the tetramine spermine and the diamines putrescine and cadaverine partially inhibited enzyme activity at the concen-

trations used. The basic amino acids D- and L-histidine had no effect on ATPase activity.

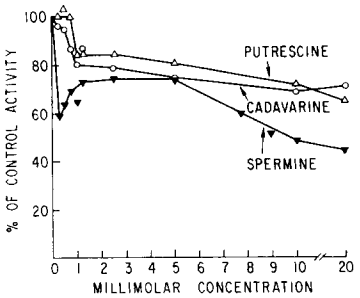

Fig. 2. Effects of spermine, putrescine, and cadaverine on the Mg^{2+}-membrane ATPase activity.

Comparison of the effects of various membrane active agents on membranes and enzyme activity

The data summarized in Table I compare the activation of ATPase by membrane active agents with release of activity from the membranes. Sodium deoxycholate and phospholipase A caused stimulations of the enzyme that were accompanied by disruption of membrane structure when observed by phase contrast microscopy. Significant amounts of activity and protein were released by these treatments. Stimulation mediated by β-lysin was not accompanied by release of activity from the membranes or by any detectable change in membrane structure. Release of protein was not determined. The cationic detergent also stimulated activity without release of enzyme or measurable protein to the supernatant. Small clumps of membranes were seen after CTAB treatment.

TABLE I

COMPARISON OF ATPase ACTIVATION WITH RELEASE OF ENZYME AND PROTEIN

Membranes were preincubated with each substance listed for 30 min in 0.075 M Tris–HCl buffer (pH 7.2) (0.15 M was used for β-lysin). Portions of the preincubates were centrifuged at 30 900 · *g* at 20 °C for 20 min. Aliquots of uncentrifuged preincubates and supernatants were assayed for ATPase activity at pH 7.8 and for protein.

Agent	Concn	% activation★	% activity released★	% protein released
Sodium deoxycholate	0.1 %	287	193	54
β-Lysin	0.28 mg protein/mg membrane protein	162	0	—
CTAB	1.75 mg/mg membrane protein	220	0	0
Phospholipase A	1.5 units/mg membrane protein	233	44	53

★ Percentages refer to the enzyme activity of untreated membranes.

Activation and release of ATPase from membranes by sonication

Exposure of mitochondria to high frequency sound has been reported to strip 80–97 % of the inner mitochondrial membrane particles from the membrane [18]. Such particles have recently been demonstrated in *Micrococcus lysodeikticus* to be ATPase [19].

Fig. 3 shows the effect of sonic oscillation on the distribution of ATPase activity in the supernatant fluids and membrane pellets obtained after centrifugation of the sonicated preparations at 30 900 × *g* for 30 min. Enzyme activity released into the supernatant (referred to as sonicate ATPase) increased with sonic oscillation time up to 20 s; membrane-bound activity decreased slightly with sonication time. Approx. 40 % of the membrane protein appeared in the supernatant when membranes were sonicated for 30 s. The total ATPase activity in the supernatant and membrane sediment fractions after sonication for 20 s was about 2.5-fold higher than in nonsonicated ghosts. More recent studies have demonstrated the sonicate enzyme to consist of two forms, 89.5 % of which is apparently soluble since it remained in a supernatant fraction after centrifugation at 109 000 × *g* for 30 min at 2 °C and 10.5 % which was sedimented under these conditions (Ko, M. M. and Buchanan, A. M., unpublished observations).

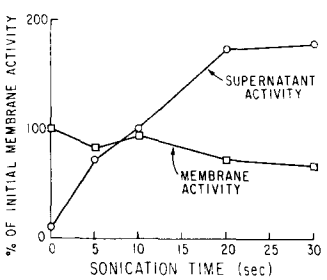

Fig. 3. Effect of sonic oscillation on membrane-ATPase activity. Three ml portions of membrane ghosts prepared from *B. subtilis* were sonicated in glass vials using the medium probe of a Biosonic III sonifier at one-half maximal output. 1-ml portions of the sonicates were centrifuged at 30 900 × *g* for 30 min. The membrane pellets were suspended in 1 ml 50 mM Tris–HCl buffer containing 5 mM $MgCl_2$. 0.1 ml of the resuspended membrane pellets and 0.2 ml of the supernatants were assayed in 0.075 M HEPES buffer (pH 7.8) for ATPase activity.

Comparison of the effects of the cationic bactericidal agents on membrane-bound and sonicate forms of ATPase

The influences of the cationic bactericidal agents on bound and on sonicate ATPase were compared. Table II shows that the sonicate ATPase was not susceptible to activation by 75 and 150 μg/ml of CTAB. Additional experiments revealed that, as with CTAB, β-lysin (48 μg protein/ml) and arginine-rich histone (100 μg/ml) did not stimulate sonicate enzyme activity. On the other hand, protamine sulfate (50 μg/ml) was found to increase both the membrane-bound and sonicate activities to approximately the same extent (180 and 220 % of the control activities, respectively). The cationic agents stimulated the ATPase activity which was sedimented at 30 900 × *g* for 30 min after sonication for 20 s (Ko, M. M. and Buchanan, A. M., unpublished observations).

TABLE II

EFFECT OF CTAB ON MEMBRANE-BOUND AND SONICATE ENZYME ACTIVITIES

ATPase obtained by sonication of isolated membranes by the method described for the experiment depicted in Fig. 3.

CTAB (μg/ml)	% of control activity	
	Membrane ATPase	Sonicate ATPase
0	100	100
75	567	133
150	197	22

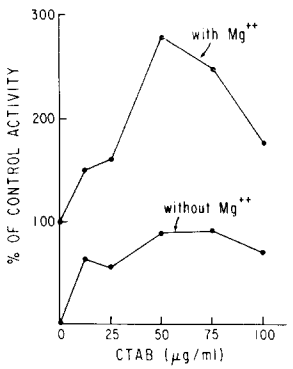

Fig. 4. Substitution of CTAB for Mg^{2+} in the enzyme assay. Results are expressed as percent of the activity with Mg^{2+} and without CTAB.

Substitution of the cationic agents for Mg^{2+} in the assay for ATPase activity

The experiment depicted in Fig. 4 was designed to determine whether CTAB could substitute for Mg^{2+} in the ATPase assay as a cation, which is necessary for enzyme activity. In the presence of Mg^{2+}, the detergent activated membrane ATPase to a high level, as one would expect, but it also stimulated membrane ATPase in the absence of Mg^{2+}. CTAB in concentrations of 40–75 μg/ml stimulated ATPase to almost the normal Mg^{2+}-stimulated level. Unlike CTAB, β-lysin, histones, and protamine sulfate could not replace Mg^{2+} in the assay.

Inhibition by anionic phospholipids of the effects of CTAB on membrane ATPase

The anionic phospholipids phosphatidylethanolamine (Fig. 5a) and phosphatidyl-L-serine (Fig. 5b) reacted with CTAB and membranes in a complex manner and blocked the activation and inhibition of membrane ATPase by CTAB (see Fig. 1b) at specific ratios of detergent to phospholipid.

The curves obtained with membranes treated with three concentrations of CTAB and increasing amounts of phosphatidylethanolamine are illustrated in Fig. 5a. Phosphatidylethanolamine added alone had very little influence on the ATPase at concentrations between 0 and 250 μg/ml. Molar equivalent concentrations (in μg/ml) were calculated assuming molecular weights of 560 and 365 for phospholipid and

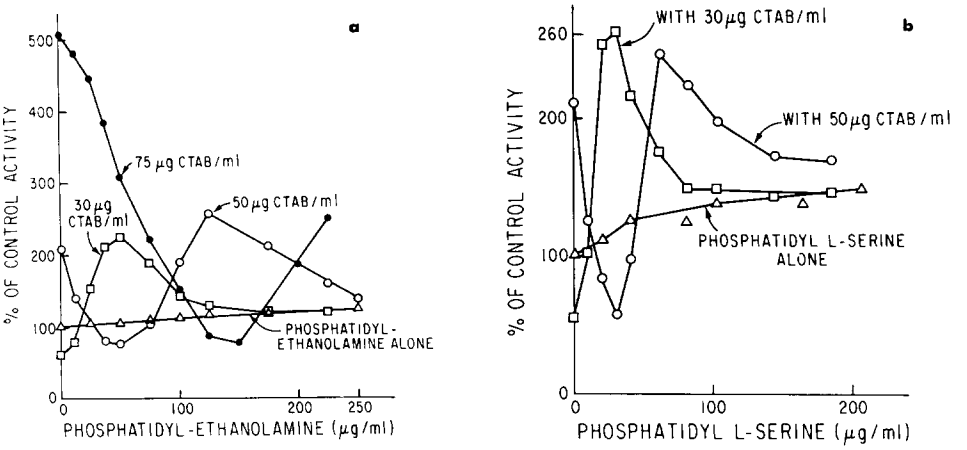

Fig. 5. Inhibition by anionic phospholipids of the influences of various concentrations of CTAB on membrane ATPase. a, phosphatidylethanolamine; b, phosphatidyl-L-serine. Detergent and phospholipid were added to the assay tube first, followed by substrate and the membrane preparation. Control activity (100 %) refers to the activity obtained in the absence of CTAB and phospholipid. △, phospholipid alone; □, with 30 μg CTAB/ml; ○, with 50 μg CTAB/ml; ●, with 75 μg CTAB/ml.

CTAB, respectively. Inhibition (60 % of control activity) by 30 μg/ml CTAB (or 82.2 μM) was blocked by the molar equivalent of phosphatidylethanolamine (46 μg/ml). Concentrations of phospholipid greater than this level progressively reduced the secondary CTAB + phospholipid-mediated activation such that the activity approached that due to phospholipid alone.

Stimulation by 50 μg/ml CTAB (208 %) was maximally blocked by phosphatidylethanolamine in the range 25–75 μg/ml; the molar equivalent of this latter agent being 75 μg/ml. Concentrations of phosphatidylethanolamine between 75 and 125 μg/ml permitted secondary stimulation of ATPase activity to 260 %, while concentrations of phosphatidylethanolamine beyond 125 μg/ml steadily reduced this latter enzyme activation such that the curve approached that of the phospholipid alone.

CTAB at 75 μg/ml stimulated enzyme activity to 507 % of the control. This stimulation was maximally blocked by the molar equivalent concentration of phosphatidylethanolamine, 125 μg/ml. As observed with the intermediate concentration of CTAB (50 μg/ml), concentrations of phosphatidylethanolamine beyond the blocking range caused a marked secondary stimulation of enzyme activity.

Alteration of CTAB-mediated inhibition and stimulation of ATPase by phosphatidyl-L-serine is illustrated in Fig. 5b. The data are essentially the same as with the phosphatidylethanolamine studies with two exceptions. Only 1/2 the molar equivalents of phosphatidyl-L-serine were needed to block CTAB-mediated inhibition and activation of membrane ATPase. Increasing amounts of phosphatidyl-L-serine resulted in a gradual but modest stimulation. If it is assumed that the anionic phospholipids reacted with CTAB via coulombic interaction resulting in neutralization of charges, then this observation is to be expected since phosphatidylethanolamine is mononegatively charged and phosphatidyl-L-serine is binegatively charged. Like the experiments with phosphatidylethanolamine, concentrations of phosphatidyl-L-serine

exceeding the amount required to reverse the effects of 50 μg/ml of CTAB caused a secondary stimulation of the enzyme which was reversed with additional phospholipid.

DISCUSSION

There is in the literature some information on the interaction of cationic agents with protoplasts and/or mitochondria which suggests the existence of more than one mechanism for their killing of bacteria. Arginine-rich histone induces swelling of mitochondria [20] with a stimulation of the mitochondrial ATPase [21] and increases in the consumption of O_2 and the efflux of K^+ [22]. Histones and protamines are lethal for *Escherichia coli*, inhibiting respiration and inducing leakage of amino acids [23]. The detergent, CTAB, induces leakage of 260-nm absorbing material as well as of glutamic acid and inorganic phosphorus from treated cells [14]. Contact with β-lysin results in a rapid rupture of protoplasts [10, 11]. On the other hand, the tetramine, spermine, stabilized protoplasts against osmotic shock [15, 16] and inhibited the transport of neutral aliphatic amino acids into cells of *M. lysodeikticus* [24].

Evidence supporting multiple mechanisms for action can be seen in the present data. β-Lysin, histones, CTAB, and protamine sulfate stimulated the membrane ATPase of *B. subtilis* (Fig. 1), while spermine inhibited it (Fig. 2). CTAB was an effective substitute for Mg^{2+} in forming an acceptable substrate complex with ATP (Fig. 5), while histones and β-lysin were ineffective. Protamine sulfate was the only agent tested which stimulated the released form of ATPase obtained by sonication of membranes and centrifugation at 30 900 $\times$ g.

Stimulation of ATPase could conceivably be lethal. There is general acceptance that the ATPase of the inner mitochondrial membrane and the corresponding enzyme of non-fermenting bacteria catalyze the terminal step in oxidative phosphorylation by acting in the reverse direction from that which is measured in vitro [25]. Enhanced ATPase activity could be a manifestation of a shift in the equilibrium of the reaction in vivo to a more unfavorable state for the synthesis of ATP, the net result being a defect in the synthesis of ATP. Another function of the enzyme in mitochondria, and probably also in bacteria, is to mediate the translocation of ions, Na^+, K^+, for example [23]. Stimulation of the hydrolytic activity of the enzyme would deplete the store of available ATP required by the cell for ion transport. Translocation of other nutrients which are secondarily driven by this enzyme through the gradient established between Na^+ and K^+ would be deranged, and leakage of important solutes would occur.

The solubilization of membrane ATPase by sodium deoxycholate and the failure of CTAB and β-lysin to do so are consistent with previous findings on the action of these agents on protoplasts and/or isolated membranes [10, 11, 13, 23, 26–28]. Harold pointed out that destruction of an osmotic barrier is not necessarily synonymous with gross change in morphology and suggested that CTAB, histones, protamines, and the peptide antibiotics interact with phospholipid moieties of membranes reorienting them to produce discontinuities and channels in the osmotic barrier [23]. The present work showing that such interaction results in gross effects on an important enzyme suggests that the two phenomena are inseparable, that one cannot occur without the other.

The complex curves seen with CTAB and phospholipids (Fig. 5) may be due to micellar phase changes in membrane phospholipids in the presence of various concentrations of CTAB, since this agent can also form micellar structures depending on conditions [29]. The reversal of the CTAB effects by added phospholipids cannot be due to a mere neutralization of the cation by added anion external to the membrane, because concentrations of phospholipid exceeding the blocking ranges caused secondary stimulations of the enzyme. The effect of phosphatidylethanolamine alone was minimal, and the stimulation by phosphatidyl-L-serine in the absence of CTAB was not great enough to account for this phenomenon. These observations and the fact that CTAB can substitute for Mg^{2+} in forming a complex with ATP lend credibility to a hypothesis that phospholipids and cations together have a regulatory function. Preliminary experiments with the other cationic antimicrobial agents indicate a blocking of their ATPase stimulating effects by anionic phospholipids, but in a manner less complex than seen here in the case of CTAB.

The experiments with sonicated membranes reveal that such treatment resulted in an increase in the total ATPase measurable in the preparation (sonicate plus sonicated membrane enzyme). Scholes and Smith [30] interpreted their data with *M. denitrificans* to mean that the enzymes were located on the inner surface of the membrane and that disruption made the substrates more accessible to them. The present observation that the sonicate form of the *B. subtilis* ATPase was no longer susceptible to stimulation by CTAB, histone, or β-lysin while the sonicated form of the enzyme which was sedimented by centrifugation at $30\,900 \times g$ for 30 min was susceptible to stimulation by these agents is not easily reconciled with this interpretation and suggests that a significant change in the form of the *B. subtilis* enzyme was induced by sonication.

The influence of β-lysin on ATPase was similar in every way to that of the purified histones. Anionic phospholipids have been shown to neutralize histone-mediated mitochondrial swelling [20] and stimulation of mitochondrial ATPase [21], as well as the antibacterial action of β-lysin [5]. It is reasonable, therefore, to assume that this serum agent combines with anionic phospholipids, indirectly affecting the conformation (and/or regulation) of ATPase by electrostatic or steric changes in the membrane. The possibility that other enzymes are affected by such interaction is under investigation.

ACKNOWLEDGMENTS

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