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INHIBITOR STUDIES WITH ADENOHYPOPHYSEAL GRANULE MEMBRANE ATPase

EVIDENCE FOR A MEMBRANE ENVIRONMENT WHICH MODULATES SENSITIVITY TO INHIBITORS

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The limiting membranes of pituitary growth hormone and prolactin secretory granules contain a Mg^{2+}-ATPase sensitive to anions. This enzyme is in many ways similar to mitochondrial ATPase. The enzyme was potently inhibited by oligomycin (K_i $6.5 \cdot 10^{-9}$ M), and was much more sensitive to the inhibitor than pituitary mitochondrial ATPase (K_i $2.7 \cdot 10^{-7}$ M). In contrast, the enzyme activity of intact secretory granules was only sparingly inhibited by oligomycin (maximal inhibition close to 30% at $5 \cdot 10^{-4}$ M). However, oligomycin (5 μM) did diminish to basal levels the enhanced granule ATPase activity observed in the presence of a stimulatory anion (25 mM sodium sulfite). Other compounds known to inhibit the proton translocating mitochondrial ATPase were also tested for their ability to inhibit the secretory granule ATPase. A similar pattern of limited inhibition in granules and greater sensitivity in isolated membranes was seen with the inhibitors *N,N*-dicyclohexylcarbodiimide and efrapeptin. In contrast, tri-*n*-butyltin chloride was a potent inhibitor of the ATPase of intact granules, and the susceptibility of the enzyme to inhibition by this compound was less after isolation of membranes. These observations suggest that pituitary secretory granule membrane ATPase may have a proton pumping function similar to that of the mitochondrial enzyme. In addition, the data imply that the inhibitor binding site(s) may be masked, inaccessible, or ineffective in intact granules, but exposed (or activated) in isolated membranes. The greater sensitivity of granule ATPase to tri-*n*-butyltin chloride, in contrast to the greater sensitivity of membrane ATPase to the other inhibitors, indicates that the tin compound may be effective at a membrane site(s) distinct from the others, or that the mechanism of inhibition is different.

Introduction

It is well documented that the Mg^{2+}-ATPases isolated from a variety of sources including mitochondria, chloroplasts, and bacteria are sensitive to oligomycin inhibition [1,2]. These membrane-bound enzyme complexes are associated with the reversible synthesis of ATP and a proton-translocating function equated with chemiosmotic energy transduction across the membrane [3–5]. The proton-translocating function of mitochondrial ATPase is also inhibited by DCCD [6–8], and trialkyltin compounds [9,10], whereas the potent inhibitor efrapeptin may inhibit at the active site [11]. Other proton-translocating ATPases, including chromaffin granule membrane ATPase and lysosomal membrane ATPase, show differing

Abbreviations: DCCD, *N,N*-dicyclohexylcarbodiimide; SITS, 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid.

relative sensitivities to these inhibitors but little effect is noted with oligomycin [12–14].

Although sensitivity to oligomycin has commonly been taken as a marker of mitochondrial origin for Mg^{2+}-ATPases, some ATPase preparations of non-mitochondrial origin are also somewhat inhibited by oligomycin. The erythrocyte anion sensitive ATPase [15] and plasma membrane ($Na^+ + K^+$)-ATPase [16,17], though inhibited by the antibiotic, are considerably less sensitive than mitochondrial ATPase. Hence, although caution should be observed in the assignment of mitochondrial origin based on oligomycin sensitivity, nonetheless highly oligomycin-sensitive ATPases have not been identified in extra-mitochondrial sites.

In the present report, we describe marked changes in the inhibitor sensitivity of pituitary secretory granule membrane Mg^{2+}-ATPase. This membrane ATPase is relatively insensitive to oligomycin when tested in intact secretory granules; in this physical form, it is also relatively resistant to inhibition by DCCD, and efrapeptin. When granules are disrupted by hypotonic lysis, the subsequently isolated membranes [18,19] display a remarkable increase in sensitivity to both oligomycin and DCCD. In the case of oligomycin, the resulting ATPase is in fact 40-fold more sensitive than the pituitary mitochondrial ATPase. A more moderate increase in sensitivity is noted for efrapeptin whereas less inhibition is observed with tri-*n*-butyltin chloride.

Experimental procedures

Materials. Frozen porcine or bovine pituitaries were obtained from Pel-Freez, Inc., Rogers, AR. No differences in ATPase activity between porcine and bovine or fresh and frozen secretory granules have been noted [19]. Sodium ATP, oligomycin, tri-*n*-butyltin chloride, and vanadate were purchased from Sigma Chemical Co. while DCCD was from ICN. Efrapeptin was a gift from Dr. Robert L. Hamill of the Lilly Research Laboratories. All other reagents were obtained from commercial suppliers at the highest available grade. Inhibitors were dissolved in 95% ethanol; the concentration of ethanol was $< 2\%$ in the enzyme reactions and did not interfere.

Methods. Preparation of pituitary subfractions. Highly purified large secretory granules containing growth hormone and prolactin were prepared by differential and sucrose density gradient centrifugation as previously described [18,19]. Secretory granule membranes were prepared by hypotonic lysis (20-fold dilution in 0.01 M Tris-HCl, pH 7.4 for 2 h at 4°C) followed by sucrose density gradient centrifugation as described [19]. Storage of membranes was at −70°C in 0.05 M Tris-HCl/0.3 M sucrose, pH 7.4.

The relative purity of the granule and granule membrane fractions has been previously established by electron microscopy and marker enzyme assays to be of the order of 92–95% [19]. Mitochondrial and plasma membrane fractions were obtained as described [19].

ATPase assay. Routine assay conditions were, in 0.5 ml: 0.05 M Tris-HCl (pH 8.0), 2.5 mM Na_2ATP, 2 mM $MgCl_2$, 5 mM KCl, 0.25 M sucrose, and 100–250 μg secretory granule, 15–60 μg secretory granule membrane, or 15–60 μg mitochondrial fraction protein. Reactions were started by the addition of enzyme after a 10 min preincubation at 37°C; termination was by the addition of 0.5 ml cold 10% trichloroacetic acid. After centrifugation, inorganic phosphate was determined by a modification of the method of Hurst [20] as previously described [19]. Enzyme units are expressed as nmoles phosphate liberated per minute at 37°C. Protein was determined by the method of Lowry et al. [21].

Results

We have previously reported the presence of an anion-sensitive Mg^{2+}-ATPase in the pituitary secretory granule membrane [19]. The specific activity of the membrane ATPase in seven separate preparations was 138 ± 18.6 units/mg. The stimulatory anions included sodium bicarbonate (35–40% increase in activity with 50 mM) and sodium sulfite (80–110% increase in activity with 25 mM).

A comparison of the effect of oligomycin on the secretory granule membrane ATPase and that in other pituitary subcellular fractions is shown in Fig. 1. As is apparent, the ATPase in the intact secretory granule fraction is relatively refractory to

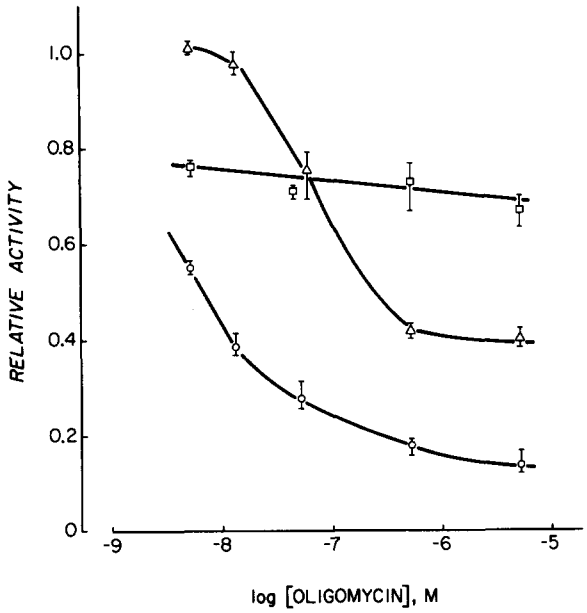

Fig. 1. Oligomycin inhibition of Mg^{2+}-ATPase activity of adenohypophyseal subcellular fractions. Brackets indicate the range of quadruplicate determinations at each oligomycin concentration. Activities in the absence of oligomycin, set at 1.00 for each fraction, were 16.5 units/mg protein for secretory granules, 70.5 units/mg for secretory granule membranes, and 151 units/mg for mitochondria. The amount of protein used was 240 μg for secretory granules (□——□), 20 μg for secretory granule membranes (○——○), and 80 μg for the mitochondrial fraction (△——△). The data shown are from one experiment, representative of six oligomycin experiments performed.

the inhibitor. Even concentrations as high as $5 \cdot 10^{-4}$ M (not shown in the figure) produced only 28–30% inhibition. Surprisingly, however, the isolated granule membranes demonstrated extreme oligomycin sensitivity. As seen in the figure, the membranes were even more sensitive than the mitochondrial fraction. For example, although there was no inhibition of the mitochondrial ATPase at an oligomycin concentration of $5 \cdot 10^{-9}$ M, this concentration resulted in 50% inhibition of the secretory granule membrane enzyme activity. Inhibition time course studies (not shown) established that maximal inhibition was achieved in all fractions studied. Regardless of the mode of expression of these results, the granule membrane ATPase was always more sensitive to oligomycin (Table I). It is evident, however, that the magnitude of the difference varied: a 40-fold difference in sensitivity was observed based on inhibitor concentration, a 10-fold difference based on protein concentration, and a 4-fold difference based on enzyme units.

These experiments demonstrated the acquisition of extreme sensitivity to oligomycin when isolated granule membranes were prepared. In order to explore in more detail the possible similarities and differences among the ATPase preparations, we next examined the interaction between oligomycin and stimulatory anions. The results of these experiments are shown in Table II. Addition of 25 mM sulfite to the reaction mixtures resulted in approximately a 2-fold increase in all three tested fractions. Oligomycin at $5 \cdot 10^{-6}$ M caused 66% inhibition of the mitochondrial ATPase and 67% inhibition of the secretory granule membrane ATPase, but only a 27% inhibition of the enzyme in intact granules. When sulfite and oligomycin

TABLE I

OLIGOMYCIN SENSITIVITY OF SECRETORY GRANULE MEMBRANE ATPase AND MITOCHONDRIAL ATPase

Maximal inhibition determined at $5 \cdot 10^{-5}$ M oligomycin. Ratios of concentration required for half-maximal inhibition, mitochondria/secretory granule membrane; 38.5, 10.2, 3.9, respectively, for K_i, ng/μg protein, and ng/units·mg^{-1}.

Enzyme fraction	K_i (M)	50% inhibition of activity		Maximal inhibition (%)
		[oligomycin] / [protein] (ng/μg) ratio	[oligomycin] / ATPase activity (ng/(nmol·min^{-1}·mg^{-1})) ratio	
Secretory granule membrane	$6.5 \cdot 10^{-9}$	0.064	0.91	82
Mitochondria	$2.5 \cdot 10^{-7}$	0.65	3.5	66

TABLE II

RESPONSES OF PITUITARY Mg^{2+}-ATPase TO SODIUM SULFITE AND OLIGOMYCIN

Results are presented as the mean and the range of quadruplicate determinations, and are relative to the control for each fraction. Control values were : secretory granule membranes, 52 units/mg; secretory granules, 14.2 units/mg; and, mitochondria, 91.4 units/mg.

Incubation condition	Relative Mg^{2+}-ATPase activity		
	Secretory granule membranes	Secretory granules	Mitochondria
No additions	1.00 (0.98–1.01)	1.00 (0.92–1.10)	1.00 (0.97–1.03)
5 μM oligomycin	0.33 (0.32–0.35)	0.73 (0.70–0.77)	0.34 (0.33–0.37)
25 mM sodium sulfite	2.38 (2.30–2.41)	1.73 (1.70–1.88)	1.85 (1.84–1.88)
5 μM oligomycin + 25 mM sodium sulfite	0.56 (0.51–0.69)	0.92 (0.89–0.94)	0.46 (0.44–0.46)

were combined, the resulting ATPase activity of intact granules was comparable to the control value (91.6% of control), whereas both the mitochondrial (45.7%) and the granule membrane enzymes (56.3%) retained only about one-half of their control activities. Compared to results obtained with sulfite alone, secretory granules exposed to both sulfite and oligomycin were inhibited by 47% while granule membranes and mitochondria were inhibited by 75%.

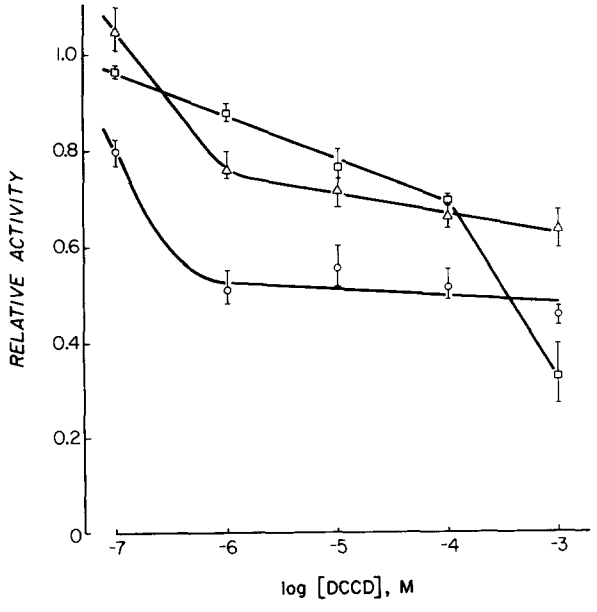

Fig. 2. DCCD inhibition of pituitary Mg^{2+}-ATPase. Brackets indicate the range of quadruplicate determinations. Control activities for each fraction were as follows: 20 units/mg for the 180 μg of secretory granules (□———□); 91.5 units mg for the 25 μg of secretory granule membranes (○———○); and 116 units/mg for the 180 μg of mitochondrial fraction (△———△) used. Data shown are from one representative experiment; three DCCD experiments were performed, and gave similar results.

Inhibition by DCCD was also compared in the pituitary subfractions to ascertain whether the secretory granule membrane ATPase was susceptible to this inhibitor and, if so, how this compared to the inhibition of either mitochondrial ATPase or the enzyme of intact granules. Fig. 2 depicts the dose response curves for DCCD inhibition. Like

TABLE III

INHIBITION OF PITUITARY ATPases BY EFRAPEPTIN AND TRI-*n*-BUTYLTIN CHLORIDE

Values given in the table are averages of four determinations. The enzyme specific activities were 5.4 ± .4 units/mg (mean ± S.E., $n = 6$) for secretory granules, 59.9 ± 3.9 units/mg ($n = 6$) for secretory granule membranes, and 99.6 ± 2.0 units/mg ($n = 5$) for the mitochondrial enzyme.

Inhibitor (μg/ml)	ATPase activity (% inhibition of control)		
	Secretory Granules	Secretory granule membranes	Mitochondrial fraction
Efrapeptin,			
4.3	22.9	51.3	37.1
21.4	23.9	49.6	44.7
42.8	37.5	50.8	45.7
Tri-*n*-butyltin chloride			
6.4	17.4	9.4	17.6
64.3	63.7	22.0	42.9
128.6	68.3	43.1	46.4

the results obtained with oligomycin, inhibition with DCCD was enhanced in granule membranes, and the granule membrane ATPase was more sensitive to inhibition than mitochondrial ATPase.

In addition to DCCD and oligomycin, other compounds known to inhibit mitochondrial ATPases were tested for inhibition of enzyme activity in the pituitary fractions. Results with efrapeptin and tri-*n*-butyltin chloride are shown in Table III. Increased sensitivity to efrapeptin was also observed upon isolation of granule membranes, maximum inhibition (51%) being observed at 4.3 μg/ml. At this concentration, activity in intact granules was only 23% inhibited. In contrast to these findings, tri-*n*-butyltin was a more potent inhibitor of the granule enzyme than the ATPase of either granule membranes or mitochondria (64% inhibition compared to 22% and 43%, respectively), at a dose of 64 μg/ml).

In order to establish conclusive functional differences not only between granule membrane and mitochondrial ATPase but also between granule membrane and plasma membrane ATPase, we examined the effects of ouabain and vanadate on the granule membrane enzyme. Neither $(Na^+ + K^+)$-ATPase activity nor any inhibitory effect of ouabain (at 0.1 mM and 1.0 mM) was found. Vanadate at 50 μM, which reduced plasma membrane ATPase activity by half, had only a small (20%) inhibitory effect on the granule membrane enzyme.

Discussion

ATPases can be characterized functionally by their involvement in proton translocation or their participation in a cation-pump mechanism. Although there are some enzymes which share characteristics from both categories [22], in general the former enzymes are characterized by a complex multi-subunit organization inhibited by oligomycin, while the latter enzymes are sensitive to vanadate with demonstrable reversible phosphorylated protein intermediates [23]. The identification of a $(HCO_3^- + Mg^{2+})$-ATPase in erythrocyte plasma membranes [15] constituted an unequivocal demonstration that an anion-activated ATPase could exist extramitochondrially. Although this enzyme was inhibited by oligomycin [24], other properties suggested a close relationship to the plasma-membrane $(Ca^{2+} + Mg^{2+})$-ATPase [24,25]. Prior to the identification of this enzyme, the question had arisen whether anion-sensitive activities found in plasma membrane and microsomal fractions were only the reflection of mitochondrial contamination (reviewed in Ref. 26). Inhibitor studies, phospholipid composition analyses, and molecular weight determinations of solubilized ATPase fragments suggested that mitochondrial contamination accounted at least in part for some of these characteristics [26].

As noted above, the granule membrane enzyme differs from the cation-pump pituitary ATPase not only in subcellular location but also in many chemical properties. It is, however, similar to mitochondrial ATPase. This similarity is not due to mitochondrial contamination. We have previously shown that the mitochondrial ATPase contamination of our granule membrane fraction is only 4–8% [19], indicating that the anion-sensitivity and other characteristics of this ATPase are intrinsic to the granule membrane enzyme. It is pharmacologically similar to mitochondrial ATPase, demonstrating both anion stimulation and inhibition by blockers of mitochondrial ATPase proton translocation. Chemical similarity between the enzymes is also suggested by the striking parallelism of the inhibitory dose-response curves with both oligomycin and DCCD (Figs. 1 and 2). Further, the observed patterns of activity in the combined presence of oligomycin and sulfite (Table II), or with efrapeptin (Table III), are similar. However, the granule membrane enzyme is considerably more sensitive to oligomycin than the mitochondrial enzyme (Fig. 1, Table I). Further, the aggregate data on relative potencies and patterns of inhibition of efrapeptin and tri-*n*-butyltin chloride on granule, granule membrane, and mitochondrial ATPases are distinct from one another.

Perhaps the most dramatic finding of the present study was the enormous change in sensitivity to oligomycin which occurred when membranes were prepared from intact granules. Similar but smaller changes in sensitivity to DCCD and efrapeptin were noted. These marked changes in responsivity warrant an examination of the factors known to be capable of influencing sensitivity to oligomycin. Extraction of ATPase-containing

membranes under suitable conditions [27–30] results in an enzyme complex still inhibited by the antibiotic. However, solubilization of the ATPase activity by removal of both the oligomycin-binding protein in the 'membrane sector' and the protein-stalk fragment results in loss of sensitivity [31,32]. Other factors influencing enzyme susceptibility to oligomycin can be divided into two categories. The first includes those involved in the conformation of the oligomycin-sensitive site and its orientation in the membrane. Included here would be the concentrations of H^+ and K^+ [33] and also the composition of the phospholipid environment [34–37]. The second category is related to the proton translocating function of the enzyme, the proposed site of oligomycin inhibition [38,39]. Provision of alternate membrane channels with the K^+-proton ionophores nigericin or valinomycin effectively circumvents the oligomycin-induced block [39].

The present data suggest that the ATPase in intact granules is in some way protected from oligomycin. Since the inhibitors are lipid soluble, the intra-membranous or per-membranous topography of enzyme subunits should not exert a critical influence. Possibly, these compounds are unable to bind to the proteolipid inhibitor binding site in the intact granule due to the conformation of the binding site or due to steric factors. For example, an equilibrium between a K^+-favored form and an H^+-favored form with high and low affinities for oligomycin, respectively, has been suggested to explain results with the yeast mitochondrial ATPase [33]. Such regulation of the site would not be operative in this instance, however, since the assay conditions for both secretory granule and granule membrane preparations were those favoring inhibition (pH 8.0 and 5 mM K^+). One can postulate that a blocker of the binding site has been removed during the preparation of the membranes. Since the membranes were prepared in hypotonic medium, it is possible that phospholipid factors lost during membrane isolation might have exerted a modulating influence in intact granules. It has been documented in other systems that the composition of the phospholipid environment near the inhibitor site has a dramatic influence on sensitivity [34–37]. It is also possible, however, that disruption of the three-dimensional structure of the membranes attendant upon dilution and lysis results in conformational changes which reverse charge or steric binding restrictions expressed in the intact granules. As a result, the accessibility of key regulatory membrane components is altered.

One might hypothesize that the sulfite stimulatory effect seen in these studies represents a functional uncoupling of the ATPase from an ionic gradient. However, hypotonic lysis fails to activate ATPase activity [19] in our fractions, which are prepared from frozen tissue; this may indicate an uncoupled state initially. The pharmacologically similar chromaffin granule ATPase retains sensitivity to anions and anion transport blockers even after solubilization which renders it insensitive to a proton ionophore [40], clearly indicating direct interaction of these agents with the enzyme. It seems reasonable to predict similar properties for the pituitary granule enzyme; we therefore assume the sulfite stimulation does not depend on uncoupling.

The identification of an oligomycin-sensitive ATPase in pituitary secretory granule membranes has functional implications. Oligomycin sensitivity has been equated with a proton pump mechanism; inhibition results in a block of proton transport. Pumped proton entry into granules, perhaps as a counterion for anion transport, may play a central role in granule fission and exocytotic mechanisms [41]. The interior of the anterior pituitary [42,43] and posterior pituitary [44], chromaffin (reviewed in Ref. 45), and insulin [46] secretory granule is acidic, and a transmembrane potential which is dependent upon $MgATP^{2-}$ is maintained [43,45,46]. Furthermore, SITS, an anion transport blocker, is a potent inhibitor of the pituitary secretory granule membrane ATPase we have studied [19]. However, the exact coupling between ATP hydrolysis, ion transport, and secretory events remains to be established. Despite little if any demonstrated sensitivity to oligomycin in other secretory systems, anion transport blockers and proton ionophores have been shown to block secretion in adrenomedullary cells, parathyroid cells, and platelets (reviewed in Ref. 41). The mechanism of inhibition may be related either to inhibition of transport directly, or to suppression of ATPase activity [19,41–43]. Despite the likely differences

in molecular requirements for storage of catecholamines and peptide hormones, the functional properties of the granule membranes mmay be similar. The ability of SITS to inhibit parathyroid hormone secretion [47] and of bicarbonate to stimulate prolactin secretion [48,49] are both consistent with this suggestion. Our data allow the inference that the pituitary granule membrane ATPase functions in proton translocation. Whether this is involved in hormone secretion or at another stage of the secretory granule life cycle is yet to be established.

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