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ARTICLE *in* FEBS LETTERS · MARCH 1988

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(88)80793-2 · Source: PubMed

Volume 229, number 1, 40–44 FEB 05596 February 1988

Inhibition of the H^+-ATPase in bovine adrenal chromaffin granule ghosts by diethylstilbestrol

Evidence for a tight coupling between ATP hydrolysis and proton translocation

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Received 29 December 1987

Diethylstilbestrol (DES) was found to inhibit reversibly the hydrolysis of MgATP (80% at 100 μM) and proton pump activity ($I_{50} \simeq 15$ μM, complete at 100 μM) in chromaffin granule ghosts. The parallel inhibition suggests a tight kinetic coupling between the two activities. The Mg^{2+}-ATPase activity, but not proton pumping, was partially restored by *N,N'*-dicyclohexylcarbodiimide, indicating that the two inhibitors in combination cause a partial uncoupling. The non-competitive type of inhibition shows that the action of DES is distal to the site of ATP binding and hydrolysis. Although unspecific, the interaction of DES with the chromaffin granule membrane seems primarily to affect the H^+-ATPase.

H^+-ATPase; Chromaffin granule; Diethylstilbestrol; (Bovine adrenal medulla)

1. INTRODUCTION

The H^+-ATPase (EC 3.6.1.34) of chromaffin granules from bovine adrenal medulla is known to catalyze the ATP-dependent translocation of protons across the chromaffin granule membrane [1,2]. Evidence has been presented in favour of an oligomeric structure for this enzyme [3,4], but its subunit composition and mechanism of action are still unknown. On the basis of inhibitor sensitivity it is classified among the V (vesicular or vacuolar) -type of H^+-ATPases [5]. It is insensitive to vanadate and the mitochondrial ATPase inhibitors iron-bathophenanthroline [6], azide and oligomycin, whereas NEM and DCCD completely inhibit proton translocation and 75–80% of the Mg^{2+}-ATPase activity [3,4,6–10]. NEM and DCCD have been shown to label certain polypeptides of the chromaffin granule membrane [3,4]. In addition, a vanadate-sensitive Mg^{2+}-ATPase (accounting for 20–25% of the overall Mg^{2+}-ATPase activity) has been demonstrated in chromaffin granule preparations [4,6,8].

In order to obtain further information on the coupling between ATP hydrolysis and proton translocation in the chromaffin granule H^+-ATPase, alternative inhibitors have been searched for. The nonsteroidal, synthetic estrogen DES is well known for its effects on several ionmotive ATPases [5], and its inhibition of the mitochondrial F_0F_1-ATPase has recently been described in detail [11]. Due to the functional similarities of the two ATPases, it was of interest to study the effect of DES also on the chromaffin granule H^+-ATPase. Dienestrol and hexestrol,

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Abbrevations: DES, diethylstilbestrol; DCCD, *N,N'*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; oxonol VI, bis[3-propyl-5-oxoisoxazol-4-yl]pentamethine oxonol

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00145793/88/$3.50

structural analogs of DES, were also included in the experimental series.

2. MATERIALS AND METHODS

2.1. *Materials*

Acridine orange, DES, hexestrol, and dienestrol were obtained from Sigma (St. Louis, MO). Oxonol VI was from Molecular Probes (Eugene, OR) and the detergent Chaps from Pierce (Rockford, IL). [*monoethyl*-^{3}H]DES (89.5 Ci/mmol) was from Amersham (Bucks, England). All other chemicals were of reagent grade.

2.2. *Preparation and solubilization of chromaffin granule ghosts*

Chromaffin granule ghosts, devoid of oligomycin-sensitive Mg^{2+}-ATPase activity [6], were prepared from bovine adrenal glands as described [12,13], and stored as aliquots in liquid nitrogen. In some experiments endogenous phosphatidylinositol was phosphorylated (to phosphatidylinositol 4-phosphate) by [γ-^{32}P]ATP as in [14].

Chromaffin granule ghosts (0.75 mg/ml) were solubilized by incubation for 30 min at 0°C with 1% (w/v) Chaps in 7.5 mM Hepes (pH 7.0) containing 1 mM dithiothreitol, and then centrifuged for 1 h at 100000 × *g* in a TLA 100.1 rotor (Beckman). The supernatant was assayed for Mg^{2+}-ATPase activity in the standard assay medium with 0.1% (w/v) Chaps.

2.3. *Assay of Mg^{2+}-ATPase and proton pump activity*

All assays were performed at 25°C with 5 min preincubation. Mg^{2+}-ATPase activity was measured as in [6] in a medium containing 7.5 mM Hepes (pH 7.0 with KOH), 1.2 mM $MgSO_4$, 1.2 mM Na_2ATP, and 50 mM KCl. The reaction was initiated by the addition of MgATP. In some experiments Mg^{2+}-ATPase activity was also measured spectrophotometrically at 340 nm by the coupling of ADP formation to NADH oxidation [15]. The medium (1 ml) then also contained pyruvate kinase (1 U), lactate dehydrogenase (1 U), phospho*enol*pyruvate (2.8 mM) and NADH (0.26 mM).

The generation of a pH gradient across the chromaffin granule membrane was assayed by the acridine orange method as in [6]. The experimental conditions were as described above. The generation of a transmembrane potential was assayed by dual-wavelength spectroscopy using 6 μM oxonol VI [16] and the wavelength pair 594 and 630 nm. Experimental conditions were as described above except that KCl was omitted from the medium.

2.4. *Interaction of [^{3}H]DES with the chromaffin granule membranes*

Chromaffin granule ghosts (0.15 mg/ml) were incubated in the standard assay medium with 200 μM DES (6.4×10^6 cpm) for 5 min at 25°C and centrifuged at 41200 × *g* for 30 min in a SS-34 rotor (Sorvall). The membrane pellet was extracted with chloroform/methanol (1:2, v/v) [17], and the nonpolar and polar phases, as well as the protein residue at the interface, were recovered and assayed for radioactivity. Aliquots of the nonpolar phase were dried under nitrogen and resuspended in chloroform/methanol. Thin-layer chromatography on DC-Alufolien kieselgel 60 plates from Merck (Darmstadt) was performed in *n*-hexane/diethyl ether (1:1, v/v).

2.5. *Other analytical procedures*

Dopamine β-monooxygenase activity (EC 1.14.17.1) was assayed [18] in 10 mM Mes (pH 6.0) in the presence of Chaps (0.1%, w/v) with tyramine (10 mM) as the substrate and ascorbate (0.1 mM) as electron donor.

Protein was determined according to Bradford [19] with bovine serum albumin as the standard. The kinetic parameters calculated from the data shown in fig.1B were obtained by non-linear regression analysis as described [20].

3. RESULTS

3.1. *Inhibition of Mg^{2+}-ATPase and proton pump activity by DES*

DES was found to inhibit both ATP hydrolysis and proton pump activity, measured either as the generation of a transmembrane pH gradient (fig.1A) or a membrane potential (not shown). The inhibition of proton pump activity was found to be non-competitive (fig.1B) with the estimated apparent K_m (MgATP) values of 62 ± 9 μM (uninhibited), 60 ± 2 μM (12.5 μM DES) and 60 ± 4 μM (25 μM DES). The calculated K_i values were 6.8 ± 0.3 μM (12.5 μM DES) and 3.8 ± 0.2 μM (25 μM DES).

The inhibition of the proton pump and Mg^{2+}-ATPase activities was maximal on preincubation for approx. 1 min (not shown). When DES was added after MgATP, the onset of inhibition was also rapid and the degree of inhibition comparable to that observed with membranes that had been preincubated with the inhibitor (fig.2). Dithiothreitol (1 mM) did not protect against this inhibition (fig.2), and the relative inhibition was the same when KCl was omitted from the assay medium.

3.2. *Effect of membrane solubilization*

The non-ionic detergent Chaps (1%, w/v) solubilized 38% of the granule ghost protein and 37% of the Mg^{2+}-ATPase activity. The specific activity of the solubilized Mg^{2+}-ATPase was 205 nmol $ADP \cdot min^{-1} \cdot mg^{-1}$, and the inhibition by 100 μM DES amounted to 81%.

3.3. *Reversibility of inhibition by DES*

DCCD partially reversed the inhibition of the Mg^{2+}-ATPase activity by DES over a period of time, when added after DES (fig.2). This reversal

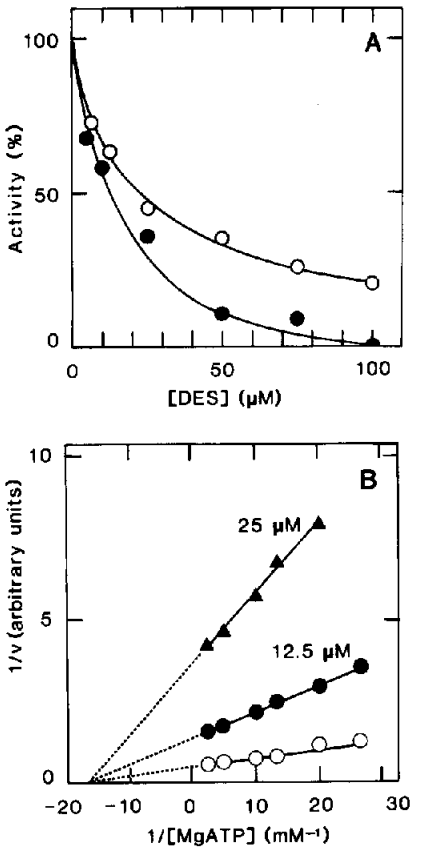

Fig.1. Inhibition of Mg^{2+}-ATPase activity and proton translocation by DES. (A) Effect of DES on Mg^{2+}-ATPase activity (○) and proton pump activity (●) of chromaffin granule ghosts. (B) Inhibition of proton pump activity (determined by the acridine orange method) by DES. The proton pump activity was measured at increasing concentrations of MgATP in the absence of inhibitor (○), and in the presence of 12.5 μM (●) and 25 μM (▲) DES. Protein, 0.047 mg/ml.

was also observed when the experiment was performed in the presence of the mitochondrial F_1-ATPase inhibitor iron-bathophenanthroline (6 μM), as in [6], or in the presence of vanadate (10 μM). The inhibition of proton pump activity was not reversed by DCCD. Bovine serum albumin, when added to the reaction mixture after DES and MgATP, almost completely reversed the inhibition of the Mg^{2+}-ATPase activity (fig.3) and proton pump activity (not shown) in native membranes. Reversal of inhibition was also observed with detergent solubilized membranes, but albumin was less effective in this case.

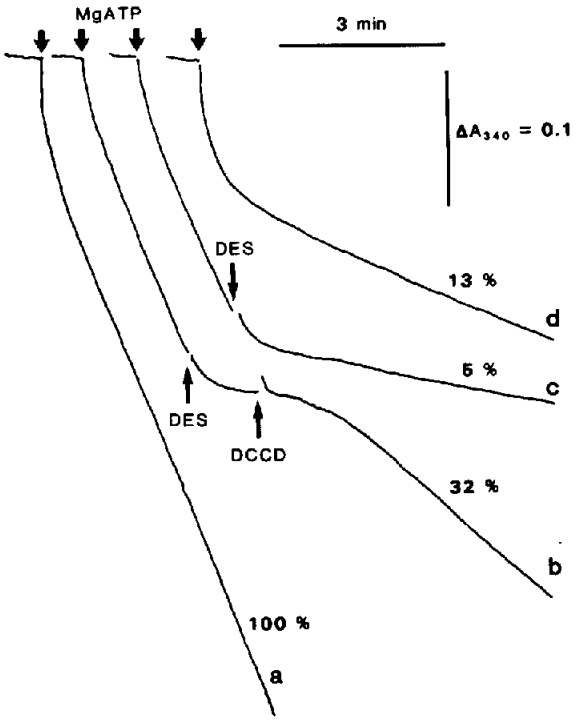

Fig.2. Time course for the inhibition of Mg^{2+}-ATPase activity by DES and its partial reversal by DCCD. Mg^{2+}-ATPase activity was measured spectrophotometrically. The standard assay medium contained in addition 1 mM dithiothreitol. Traces: a, ATP hydrolysis in the absence of added inhibitor (249 nmol ADP·min^{-1}·mg^{-1}); b, inhibition by 100 μM DES and its partial reversal by 100 μM DCCD; c, inhibition by 100 μM DES; d, inhibition by 100 μM DCCD (preincubated for 5 min). Chromaffin granule ghosts, 0.041 mg protein/ml.

3.4. *Interaction of [^{3}H]DES with the chromaffin granule membrane*

After incubation of chromaffin granule ghosts with [^{3}H]DES (see section 2), 63.8 ± 1.1% (mean ± SD, n = 3) of the radioactivity was recovered in the membrane pellet on centrifugation. After chloroform/methanol extraction of the pellet, 98.6 ± 0.2% of the radioactivity was recovered in the nonpolar phase and 0.097 ± 0.02% remained in the protein residue at the interface. On TLC analysis of the nonpolar phase 90.3% of the radioactivity migrated as *cis*- or *trans*-DES, as compared to 93.7% in the isotope stock solution. 7.5% of the radioactivity was found in an unidentified spot located between those corresponding to the *cis* and *trans* forms of DES.

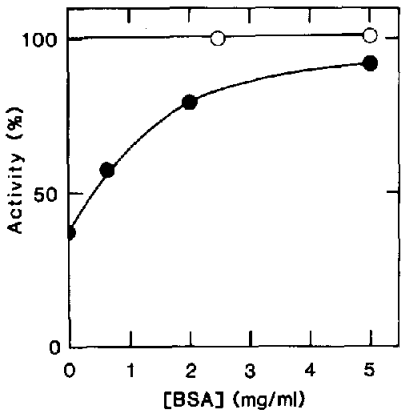

Fig.3. Reversal of the DES inhibition of Mg^{2+}-ATPase activity by increasing concentrations of bovine serum albumin (BSA). Mg^{2+}-ATPase activity was measured spectrophotometrically in the absence of added inhibitor (○), and in the presence of 100 μM DES (●). When the Mg^{2+}-ATPase activity reached a steady state, bovine serum albumin was added, and the activity in the new steady state was measured. 100% represents the activity in the absence of added DES and albumin, i.e. 200 nmol ADP·min^{-1}·mg^{-1}. Chromaffin granule ghosts, 0.041 mg protein/ml.

3.5. *Inhibition by dienestrol and hexestrol*

Dienestrol and hexestrol were also potent inhibitors of the Mg^{2+}-ATPase and proton pump activities (table 1).

3.6. *Other investigations*

Incubation of chromaffin granule ghosts with DES (100 μM) did not cause any significant release of membrane proteins or lipids (measured as the release of ^{32}P-labeled phosphatidylinositol 4-phosphate), and the redox state of cytochrome *b*-561 in native membranes was not affected. Dopamine β-monooxygenase activity was not affected by DES.

Table 1

The effect of diethylstilbestrol and two analogs on the proton pump activity of chromaffin granule ghosts

Inhibitor[a]	I_{50} (μM)	I_{100} (μM)
Diethylstilbestrol	15	100
Dienestrol	30	100
Hexestrol	15	150

[a] Diethylstilbestrol, α,α'-diethylstilbenediol; dienestrol, 4,4'-(diethylideneethylene)diphenol; hexestrol, 4,4'-(1,2-diethylethylene)diphenol

Proton pump activity was measured by the acridine orange method. I_{50} and I_{100}, concentrations of the inhibitor giving 50 and 100% inhibition, respectively, the values being obtained from inhibitor titrations as in fig.1A

4. DISCUSSION

In the present study it is shown that DES is a potent inhibitor of membrane associated (fig.1) and detergent-solubilized chromaffin granule H^+-ATPase. The inhibition was observed in the same concentration range as DES inhibits the mitochondrial F_0F_1-ATPase [11] and the plasma membrane H^+-ATPase of *Neurospora crassa* [21]. The Mg^{2+}-ATPase activity remaining (approx. 20% of the total) after complete inhibition of proton translocation is in good agreement with our estimates on the contribution of the vanadate-sensitive Mg^{2+}-ATPase activity in chromaffin granule ghosts [6].

In contrast to NEM and DCCD [3,4], DES seems to be a non-covalently interacting inhibitor of the H^+-ATPase. The DES molecule does not contain functional groups which can react with amino acid residues, and studies with [^{3}H]DES did not demonstrate any metabolic conversion of the compound by the granule membrane. The partitioning of membrane-associated [^{3}H]DES into the nonpolar phase on chloroform/methanol extraction indicates that the inhibitor is located in the most hydrophobic parts of the membrane. The addition of bovine serum albumin to the assay medium increases the number of hydrophobic binding sites available for the inhibitor, and the reversal of inhibition of the Mg^{2+}-ATPase probably reflects a decrease in the concentration of DES in the chromaffin granule membrane.

At high concentrations DES has been shown to possess a membrane-perturbing effect in liposomes [22], and it is possible that the inhibition of the chromaffin granule H^+-ATPase is due to an unspecific perturbation of the granule membrane structure and the microenvironment of the enzyme. This possibility is supported by the fact that the compound also inhibits the phosphatidylinositol kinase activity (EC 2.7.1.67), another MgATP-requiring enzyme of the granule membrane, but with less potency ($I_{50} \simeq 120$ μM) (Husebye, E., personal communication). The low K_i obtained for in-

hibition by DES, as well as its lack of effect on a number of other membrane-associated activities, suggest that inhibition of the H^+-ATPase is not due solely to an unspecific membrane effect.

The parallel inhibition of ATP hydrolysis and proton translocation by DES demonstrates a tight kinetic coupling between the two activities, and differs from what we have observed for the inhibition by NEM [6,7]. The strictly non-competitive type of inhibition indicates that the action of DES is distal to the site of ATP binding and hydrolysis. The partial reversal of the inhibition of Mg^{2+}-ATPase activity, but not proton pumping, by DCCD indicates that DCCD and DES in combination cause a partial uncoupling of the H^+-ATPase. A similar type of uncoupling has been observed for the mitochondrial F_0F_1-ATPase in which DCCD completely reverses the inhibition of the ATPase activity by DES [11]. In this case, DCCD is known to bind specifically to a proteolipid in the F_0 part (proton channel), and it is of interest that a DCCD-binding proteolipid has also been reported as part of both the chromaffin granule H^+-ATPase [8] and its closely related counterpart in clathrin coated vesicles [23]. The results presented here provide additional support for the proposal [8] that the oligomycin-insensitive chromaffin granule H^+-ATPase has mechanistic features in common with the mitochondrial F_0F_1-ATPase.

Acknowledgements: The technical assistance of Ms Randi Svebak is gratefully acknowledged. This work was supported by the Norwegian Research Council for Science and the Humanities, the Norwegian Council on Cardiovascular Diseases, Nansenfondet and the Nordic Insulin Foundation.

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