# Inhibition by *N*-ethylmaleimide of the MgATP-driven proton pump of the chromaffin granules

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The thiol reagent *N*-ethylmaleimide (NEM) completely inhibits the proton pump activity of the $H^+$-ATPase in chromaffin granule 'ghosts' at concentrations which only partly (~20%) inhibit the $Mg^{2+}$-dependent ATP hydrolysis. Half-maximal inhibition was obtained at ~13 $\mu$M NEM as compared to 18 $\mu$M for the classical proton channel inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD), and the apparent stoichiometry of the inhibitors at complete inhibition was NEM:DCCD ≃ 1:2. High concentrations of NEM (>100 $\mu$M) induce a dissipation of the transmembrane potential generated by MgATP. These findings establish NEM as a valuable proton channel inhibitor in chromaffin granules and explain the rather complex effect of NEM previously reported for catecholamine accumulation in this organelle.

*Proton pump* *Proton channel* *$H^+$-ATPase* *Chromaffin granules*
N-*ethylmaleimide* *Adrenal medulla*

## 1. INTRODUCTION

Chromaffin granules possess a very effective mechanism of uptake of biogenic amines driven by an electrochemical proton gradient ($\Delta\mu_{H^+}$) generated by an $H^+$-ATPase located in the granule membrane [1–9]. The vectorial movements of protons (from the cytosol) results in the generation of a $\Delta$pH (inside acidic) and a $\Delta\psi$ (inside positive) [1–6]. Amine accumulation is thought to proceed by a separate carrier-mediated process, inhibitable by reserpine [10] and dependent upon both components of $\Delta\mu_{H^+}$ (review [11]).

Uptake of catecholamines is inhibited by thiol reagents such as *N*-ethylmaleimide (NEM) [12–15]; the effect of NEM is rather complex with a differential effect on the influx and the efflux when measured as a function of concentration [14,15]. Although a correlation has been found between amine uptake and ATPase activity [12,13], concentrations of NEM, which completely inhibit amine uptake, cause a variable (0–100%) inhibition of the $Mg^{2+}$-ATPase activity depending on [MgATP] [13]. Thus, the stoichiometry and the degree of coupling between ATP hydrolysis and catecholamine uptake is not yet clear.

Here we show that low concentrations of *N*-ethylmaleimide completely inhibit the proton-pumping activity of the $H^+$-ATPase, whereas higher concentrations induce a dissipation of the membrane potential generated by MgATP-hydrolysis. In addition, experimental evidence is given for a distinction between the proton pump activity and the capacity of the granule 'ghosts' to catalyze ATP hydrolysis.

*Abbreviations*: ANS, 1-anilinonaphthalene 8-sulphonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon; $\rightarrow H^+$, quantity of acid equivalents translocated inwards; NEM, *N*-ethylmaleimide; $\Delta$pH, pH difference across the membrane; PIPES, piperazine-*N,N*-bis-2-ethanesulphonic acid; $\Delta\psi$, transmembrane potential

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

ATP, dithiothreitol and *N*-ethylmaleimide were obtained from Sigma (St Louis MO), *N,N'*-dicyclohexylcarbodiimide from Koch-Light Ltd. (Colnbrook), 1-anilinonaphthalene 8-sulfonic acid from

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Eastman (New York) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone from Boehringer (Mannheim).

### 2.2. *Preparation of chromaffin granule 'ghosts'*

Highly purified resealed chromaffin granule 'ghosts' were prepared from freshly collected bovine adrenal glands using gradient centrifugation as the final purification step [16]; 7.5 mM BES buffer, pH 7.0 at 25°C, was used as the medium for lysis and washing of the chromaffin granules. The 'ghosts' recovered from the gradient [16], were stored in liquid nitrogen until used.

### 2.3. *Assay of ATPase activity*

The total $Mg^{2+}$-ATPase activity was assayed at 25°C in a medium containing 7.5 mM PIPES of pH 7.0, 1.25 mM MgATP (disodium salt of ATP), 2.5 $\mu$M FCCP and 1 mM $NH_4Cl$. The formation of ADP was measured as in [17].

### 2.4. *Other analytical procedures*

Protein was determined [18] using bovine serum albumin as a standard. Changes in fluorescence of the probe 1-anilinonaphthalene 8-sulphonic acid (recrystallized twice from ethanol) were measured with a spectrofluorometer (model SFM 22 from Kontron, Switzerland) with excitation at 380 nm and emission at 480 nm [1,2].

## 3. RESULTS

### 3.1. *Comparison of the total $Mg^{2+}$-ATPase activity and the proton pump activity of highly purified chromaffin granule 'ghosts'*

Properties of the total $Mg^{2+}$-ATPase activity of the highly purified granule 'ghosts' are given in table 1, when measured in preparations with no transmembrane pH gradient and membrane potential. A Michaelis–Menten saturation curve was obtained with respect to [MgATP] ($Mg^{2+}$:ATP = 1:1); the $K_M$ value (MgATP) was ~240 $\mu$M. The thiol reagent NEM was found to inhibit the total $Mg^{2+}$-ATPase activity only at high concentrations; half maximal inhibition was found at ~60 $\mu$M and the maximal inhibition (52%) was obtained at ~1 mM.

In agreement with [6], the granule 'ghosts' were well preserved with respect to the function of the proton pump (fig. 1A). Using the ANS fluorescence probe to monitor changes in transmembrane potential [1,2], the addition of 1.0 mM MgATP induced a rapid enhancement of the fluorescence ($t_{1/2} \simeq 50$ s), and a steady-state level was obtained for >5 min. The rate as well as the steady-state level of the MgATP-induced fluorescence enhancement was a saturable process; the $K_M$-value

Table 1

Comparison of some properties of the proton pump activity and the total $Mg^{2+}$-ATPase activity of the chromaffin granule 'ghosts'

| | Proton pump activity[a] | Total $Mg^{2+}$-ATPase activity[b] |
|---|---|---|
| $K_M$ for MgATP ($\mu$M) | 48 | 240 |
| [NEM] ($\mu$M) required for: | | |
| 50% inhibition | 13 | 1000 |
| Half-max. inhibition | 13 | 60 |
| 100% inhibition | 40 | n.o. |
| [DCCD] ($\mu$M) required for: | | |
| 50% inhibition | 18 | n.d. |
| 100% inhibition | 80 | n.d. |
| Inhibition by oligomycin | n.o. | n.o. |

[a] For assay, see fig. 1

[b] The activities were measured in 'ghosts' with no $\Delta$pH and $\Delta\psi$ (i.e., in the presence of 2.5 $\mu$M FCCP and 1.0 mM $NH_4Cl$) and are expressed as nmol.$min^{-1}$.$mg^{-1}$ protein

n.d., not determined; n.o., not obtained

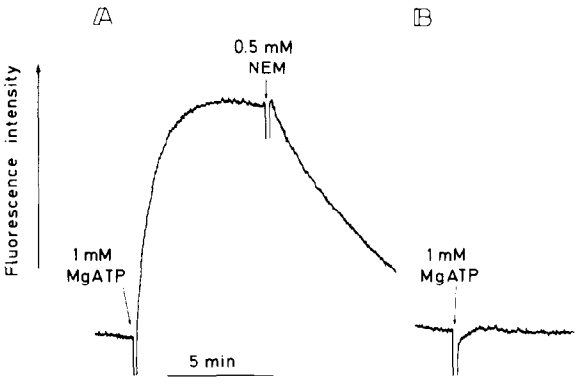


Fig. 1. The MgATP-induced increase in ANS fluorescence in chromaffin granule ghosts (A), its partial reversal by 0.5 mM NEM (A) and its complete inhibition by preincubation for 5 min with 40 $\mu$M NEM (B).

for MgATP was found to be 48 $\mu$M for the initial rate of energisation (table 1).

### 3.2. *Effect of NEM and DCCD on the proton pump activity and stability of the MgATP-induced membrane potential*

In chromaffin granules, maximally energised by 1.0 mM MgATP, the addition of a high concentration (500 mM) of the thiol reagent NEM almost completely reverses the ANS-fluorescence enhancement with a half-time of ~3.5 min (fig. 1A). Half-maximal rate of deenergisation was obtained at ~700 $\mu$M NEM (fig. 2B).

When the 'ghosts' were preincubated with 40 $\mu$M NEM before MgATP was added, no change in fluorescence intensity was observed (fig. 1B, 2A) indicating complete inhibition of $H^+$-translocation. Half-maximal inhibition of the initial rate of energisation was obtained at 13 $\mu$M, and complete inhibition was observed at ~40 $\mu$M NEM (table 1).

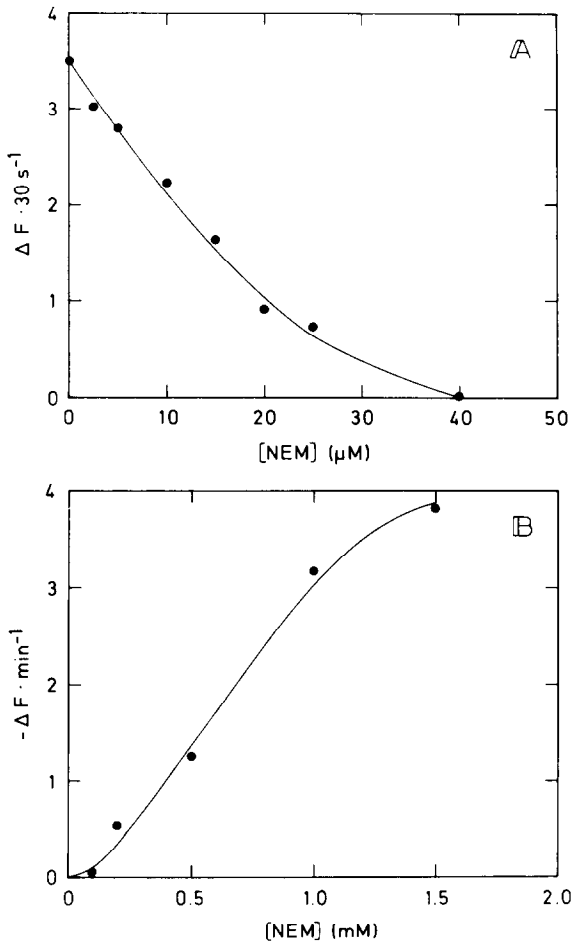


Fig. 2. Effect of NEM concentration on the generation (A) and reversal (B) of the membrane potential induced by MgATP. For experimental details see fig. 1A,B.

A characteristic feature of the $H^+$-ATPase of the chromaffin granule membrane is a high sensitivity to *N,N'*-dicyclohexylcarbodiimide (DCCD) [2]. In general, DCCD reacts specifically and covalently with a low-$M_r$ subunit (proteolipid) of the $H^+$-ATPase which is supposed to be part of the $H^+$-conducting channel [19]. A similar DCCD-reactive protein has been isolated from chromaffin granule membranes [20].

Preincubation (5 min) with ~80 $\mu$M DCCD completely inhibits the proton pump activity of chromaffin granule 'ghosts', and half-maximal inhibition was obtained at 18 $\mu$M (table 1). Although the stoichiometry in the binding of NEM and DCCD to the $H^+$-ATPase is not yet established, it is worth mentioning that the amount of inhibitors required for complete inhibition (in 1.0 ml incubation medium) was ~40 nmol NEM and ~80 nmol DCCD (table 1); i.e., a stoichiometry of NEM: DCCD $\simeq$ 1:2.

## 4. DISCUSSION

The chromaffin granule membrane contains an $H^+$-ATPase [1–9] which reversibly [21] couples the hydrolysis of ATP to the translocation of protons across the membrane. The catalytic part of the enzyme has been isolated and shown to have several features in common with the mitochondrial $F_1$-ATPase [22]. $H^+$-Translocation is inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) and a DCCD-reactive protein has been isolated from chromaffin granule membranes [20]. However, it is not inhibited by oligomycin (specific to mitochondrial $F_0$ [19]) which is experimentally very useful in eliminating any contribution by mitochondria in studies of the chromaffin granule ATPase [6].

Perhaps the most important unanswered question on the mechanism of $H^+$-ATPases in general is that of coupling. In the case of chromaffin granules, the coupling between ATP hydrolysis and $H^+$-translocation is not yet clearly established. In our determination of the $\rightarrow H^+/ATP$ stoichiometry in the granule 'ghosts', we obtained a value

of 1.58 [6]. Later measurements have suggested a value closer to 2, but even this may be an underestimate. Thus, ATP hydrolysis in granule 'ghosts' is only partly coupled to proton translocation and the degree of coupling as measured is dependent on the time interval selected for the calculations [6] as well as on [MgATP] (table 1). Thus, a distinction can be made between the two processes; e.g., on the basis of differences in $K_M$ values for MgATP. Furthermore, the thiol reagent NEM prevents the coupling event at concentrations which do not impair the capacity of the 'ghosts' to catalyze ATP hydrolysis. The results indicate that sulfhydryl groups participate in the activity of the proton channel, and that NEM may prove to be a valuable chemical modifying agent in further studies on the $H^+$-conducting pathway as well as energy coupling in this organelle.

Although the $\Delta\mu_{H^+}$ generated by the proton pump is established to be the driving force in the uptake of biogenic amines in chromaffin granules and granule 'ghosts' [1–9], there is no simple stoichiometric relationship between catecholamine transport and ATP hydrolysis, and the degree of coupling is not yet clear [23]. Furthermore, concentrations of the thiol reagent NEM which completely inhibit uptake, cause a variable (0–100%) inhibition of the $Mg^{2+}$-ATPase activity [13]. The effect of NEM observed here thus of interest: NEM inhibits (on preincubation) the total $Mg^{2+}$-ATPase activity only at high concentrations (half-maximal inhibition at ~60 $\mu$M and only ~50% maximal inhibition) whereas it inhibits proton translocation at lower concentrations (half-maximal inhibition at 13 $\mu$M and 100% maximal inhibition). This finding explains reports that concentrations of NEM, which completely inhibit the uptake of catecholamines driven by $\Delta\mu_{H^+}$, cause only a partial inhibition of the total $Mg^{2+}$-ATPase activity [13]. Furthermore, our findings offer an explanation for the rather complex effect of NEM reported for catecholamine accumulation in this organelle [14,15].

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