ATP-DEPENDENT PROTON TRANSLOCATION IN RESEALED CHROMAFFIN GRANULE GHOSTS

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1. Introduction

The catecholamine-storing organelles (chromaffin granules) of the adrenal medulla accumulate the catecholamines by a mechanism that is coupled to ATP hydrolysis [1–3] and inhibited by classical uncouplers of oxidative phosphorylation [4,5] which affect $\Delta\bar{\mu}H$ across the membrane. Evidence has recently been presented that a proton electrochemical potential difference is generated by the ATP hydrolysis [6,7], but the direction of the proton movement in intact chromaffin granules is controversial. Thus, whereas recent studies by Casey et al. [6] indicate that the direction of proton translocation is inward, Pollard et al. [7] have reached the opposite conclusion, i.e., that ATP hydrolysis causes an internal alkalinization. Since the intact granules contain a high concentration of stored compounds (catecholamines, adenine nucleotides and proteins) [8], they have a high internal buffer capacity, which in addition to leaks from the granules during an incubation, may complicate the interpretation of the results [6,9]. We have therefore studied the direction of the proton movement and its stoichiometry to ATP hydrolysis in resealed granule ghosts of low internal buffer capacity derived from bovine adrenal chromaffin granules. Using a spectrophotometric method with bromothymol blue as an indicator of intravesicular pH, it is shown that the ATP-dependent proton translocation is well preserved in the ghosts which are outside-out membrane vesicles [10], and that the direction of this translocation is inward as proposed for intact chromaffin granules [6]. It is also shown that the extent of internal acidification is dependent on the activity of the ATPase as measured by the rate of ATP hydrolysis.

Abbreviations: BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BTB, bromothymol blue; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NEM, *N*-ethylmaleimide; pH_o, pH of the suspension medium; pH_i, pH of the vesicular interior; $\rightarrow H^+$, quantity of protons translocated; $\Delta\bar{\mu}H$, proton electrochemical potential difference

2. Methods

2.1. *Preparation of chromaffin granule ghosts*

Chromaffin granules were isolated from bovine adrenal medulla as described previously [11] and granule ghosts were prepared by lysis in a hypo-osmotic salt solution containing 1 mM BES, 0.1 mM dithiothreitol, 10 μM $CaSO_4$, 2 mM $MgSO_4$, and 13 μM bromothymol blue (pH 7.5 at 35°C). The packed chromaffin granules [11] were suspended in approx. 100 vol. of this medium at 0°C by gentle homogenization [12]. The turbid suspension turned blue-green, indicating a very rapid lysis of granules. The ghost suspension was then centrifuged (37 000 × *g* for 30 min in the SS-34 rotor of Sorvall RC 5 centrifuge) and the pellet was resuspended in the same medium of pH 7.0 (at 35°C) containing no BTB, but 0.1% (w/v) bovine serum albumin which has a high binding constant for the pH-indicator [13]. The pellet of washed granule ghosts was resuspended by homogenization in a medium containing 1 mM BES, 0.1 mM dithiothreitol and 2 mM $MgSO_4$ (pH 7.0 at 35°C).

2.2. *Measurement of internal pH (pH_i) and external pH (pH_o)*

Apparent changes in intravesicular pH (pH_i) were measured by the bromothymol blue spectrophotometric method [13,14]. BTB was incorporated into the chromaffin granule ghosts during the lysis procedure (see 2.1) and transient changes in pH_i was measured by an Aminco dual-wavelength spectrophotometer using the wavelength pair 618–648 nm. The reaction medium contained 1 mM BES, 0.1 mM dithiothreitol, 2 mM $MgSO_4$ and 5 μg oligomycin (in order to inhibit any trace amount of mitochondrial ATPase activity); pH 7.0 (35°C). The pH of all solutions was adjusted to pH 7.0 (35°C). The quantity of acid equivalents translocated ($\rightarrow H^+$) was calculated by calibration of the bromothymol blue change as apparent ΔH^+ by the addition of standard acid in the presence of the proton conductor CCCP (see fig.1).

Changes in pH of the suspension medium (pH_o) were measured by a Radiometer 22 pH meter.

2.3. *Assay of ATPase activity*

ATPase activity was measured in the standard incubation medium (see 2.2) by the release of ADP as determined by high performance liquid chromatography [15].

Protein was determined using bovine serum albumin as a standard [16].

2.4. *Chemicals*

All chemicals were of reagent purity. ATP, *N*-ethylmaleimide, CCCP and oligomycin were obtained from the Sigma Chemical Company and bromothymol blue from Merck AG, FRG.

3. Results

3.1. *ATP-dependent proton translocation in chromaffin granule ghosts*

Figure 1 A is a photograph of the recorded absorbancy changes of chromaffin granule ghosts containing BTB. A large spectral change is seen immediately upon the addition of ATP (see discussion § 4.3) and is followed by a more slow exponential decrease in absorbancy at 618–648 nm. The direction of the absorbancy change indicates a decoloration of the indicator due to increased acidity within the granule

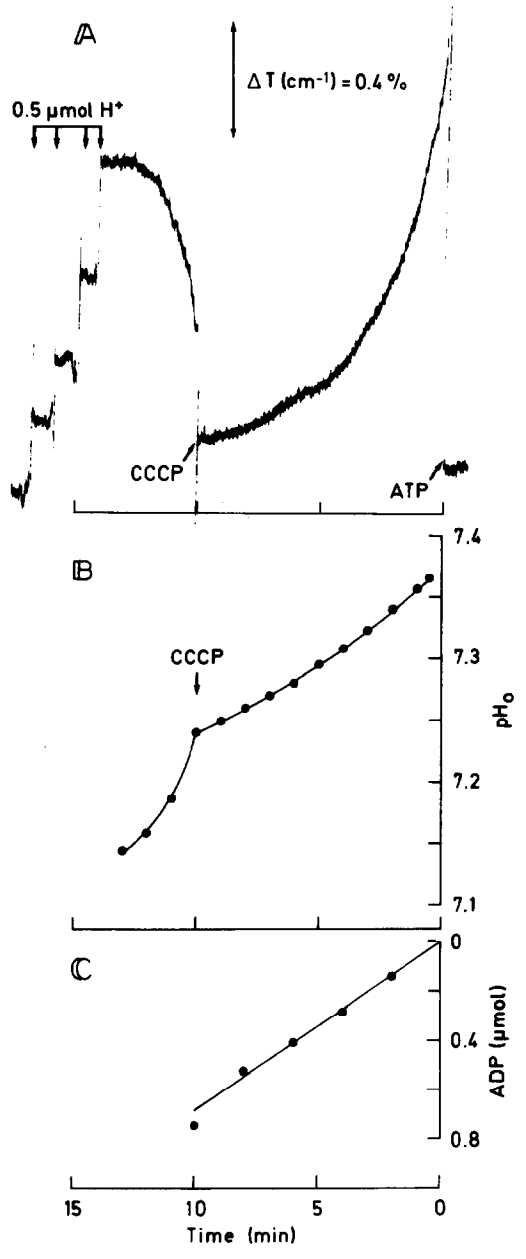

Fig.1. (A) Time-course of changes in absorbancy at 618–648 nm following addition of 10 mM ATP, 15 μM CCCP and pulses of HCl to a suspension of chromaffin granule ghosts loaded with bromothymol blue (13 μM). The ghosts (0.82 mg protein/ml) were equilibrated at 35°C at pH_o 6.8 for 5 min in 1.0 ml of a medium containing 1 mM BES, 2 mM $MgSO_4$, 1 mM dithiotreitol and oligomycin (6 μg/mg protein). (B and C) Time-course of net change in pH_o (B) and ATP hydrolysis (C) in parallel experiments to that shown in fig.1A.

space. Upon the addition of the proton conductor CCCP the trace rapidly returns to almost the initial level indicating an internal alkalinization. These spectral changes were not observed in the absence of incorporated BTB. On the other hand, as seen from fig.1B the time-course of the changes in pH_o revealed an external acidification upon the addition of the protonophore (CCCP). Thus, we may consider that the responses that are observed here refer to a net proton-uptake into the vesicles as a result of ATP hydrolysis. As expected, however, the progress curve of the ATP hydrolysis is almost linear (fig.1C) in contrast to the exponential curve of the BTB response (fig.1A).

Additions of standard acid (HCl) and base (KOH), which resulted in rapid changes in protonation of BTB bound to the outer phase of the membrane ghosts did not change the base-line slope indicating that the ghosts have a low passive permeability to protons in the pH-range studied (around pH 7.0).

3.2. *Effect of protonophores, ATP concentration and ATPase inhibitor*

The generation of a proton gradient was completely inhibited by 15 μM CCCP although the ATPase activity was significantly (approx. 50%) increased by the protonophore (fig.2). Furthermore, the extent of internal acidification ($\rightarrow H^+$) was dependent on the activity of the ATPase; i.e., dependent on the ATP concentration and inhibited by 0.5 mM *N*-ethylmaleimide (fig.3) to the same extent (approx. 80%) as the rate of ATP hydrolysis.

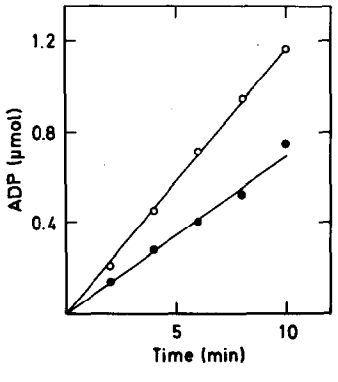

Fig.2. Time-course of ATP hydrolysis catalyzed by granule ghost ATPase in the absence (•) and presence (○) of 15 μM CCCP. Experimental conditions as in fig.1.

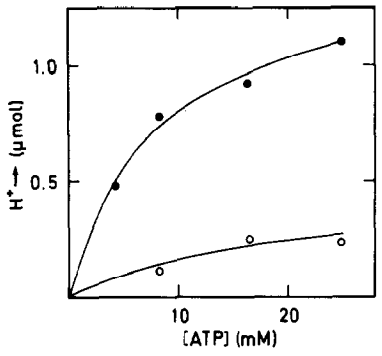

Fig.3. Effect of ATP concentration on the quantity of protons translocated ($\rightarrow H^+$) following a reaction period of 10 min (see fig.1) in the absence (•) and presence (○) of 0.5 mM *N*-ethylmaleimide. Experimental conditions as in fig.1 except that the protein concentration was 0.38 mg/ml.

4. Discussion

4.1. *Selection of method for internal pH measurements*

A number of experimental approaches have been designed in order to measure pH gradients in microscopic vesicular systems including the use of pH indicators (for review, see [17]) which enable a continuous spectroscopic monitoring of pH with fast response time and great sensitivity. In the present study bromothymol blue has been successfully used for the measurement of internal pH of chromaffin granule ghosts. This indicator is very useful near neutrality (pK_a 7.2) and it is almost exclusively located inside the particles due to an efficient washing of the vesicles in a medium containing bovine serum albumin which has a high affinity for the indicator [13]. Based on the previously reported low affinity of BTB to other biological membranes, e.g., chromatophores [18], we assume that most of the BTB in our vesicle preparation is free and reflect the intravesicular pH. No effect of BTB on the passive proton conductance of the vesicles was found at the concentrations used (13 μM).

4.2. *Selection of media*

A number of medium conditions for lysis, washing and incubation were tested. In order to obtain efficient resealing of the membrane vesicles both Ca^{2+} (10 μM) and Mg^{2+} (2 mM) ions were included in the lightly buffered medium (1 mM BES). SO_4^{2-} was selected as

the anion in all media since it is a non-penetrant in the granule membrane [6].

4.3. *ATP-dependent H^+-translocation*

It is evident from fig.1 that chromaffin granule ghosts contain a membrane-bound ATPase catalyzing the hydrolysis of external ATP which appears to be rather tightly coupled to the translocation of H^+ into the vesicles. Since the H^+-translocation is completely inhibited by the proton conductor CCCP, which increases the ATPase activity (fig.2), it is most likely that the enzyme is a (H^+)-ATPase. This conclusion is also supported by recent NMR studies on intact chromaffin granules [6]. Thus, our studies do not support the conclusion reached by Pollard et al. [7] that ATP hydrolysis leads to an internal alkalinization of these organelles.

The initial rapid increase in absorption at 618 nm upon the addition of ATP (fig.1A) is largely due to an increase in light scattering and partly due to an increase in pH_0 although the pH of the medium and the ATP solution were adjusted to $\lesssim 7.0$. A similar response has been reported in experiments of this type on submitochondrial particles [19].

4.4. *$\rightarrow H^+$/ATP ratio*

In our experiments the number of H^+-ions translocated/molecule ATP hydrolyzed by the granule ghost ATPase (the $\rightarrow H^+$/ATP ratio) varies with the reaction time selected. Following the standard reaction period of 10 min the $\rightarrow H^+$/ATP ratio was on an average found to be 1.58 ($n = 5$), which supports the conclusion that the ATPase functions as a protonmotive ATPase in the terminology of Mitchell [20]. Although measurements of initial rates give a higher $\rightarrow H^+$/ATP ratio, further studies are required in order to establish this stoichiometry in tightly-coupled membrane ghost preparations.

4.5. *Physiological function of the (H^+)-ATPase*

It has been shown that uptake of catecholamines in adrenal chromaffin granule ghosts is driven by ATP hydrolysis via a membrane-bound ATPase [1–3] and that the uptake is sensitive to agents which increase the membrane conductance of H^+-ions [4,5]. Thus, it appears most likely that the (H^+)-ATPase is responsible for this uptake, by generating an electrical potential and a pH-gradient across the membrane (positive and acidic inside) which may provide energy also for transport of other low molecular weight components found to be present in high concentrations in the granule matrix [8]. This possibility is now being tested in our laboratory.

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