ENERGY-LINKED ACTIVITIES OF THE CHROMAFFIN GRANULE MEMBRANE

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1. Introduction

The storage vesicles of the adrenal medulla (the chromaffin granules) contain unusually high levels of catecholamines (0.5 M) and nucleotides (0.125 M ATP) as well as substantial amounts of acidic protein [1]. The membrane of the chromaffin granule contains several activities that may be important in maintaining these high metabolite levels. These include an electron transport chain [2] and, in particular, an ATPase activity that has been reported to be associated with the active transport of catecholamines [3]. Earlier work [4] with the storage vesicles extracted from bovine splenic nerve has shown that uncouplers and inhibitors of mitochondrial oxidative phosphorylation can affect both the uptake and the release of noradrenaline from these vesicles. In this report we shall indicate that the ATPase activity of the chromaffin granule membrane is reflected by changes in the fluorescence of the probe ANS* and that such changes are abolished by uncoupling agents with a concomitant increase in ATPase activity.

2. Materials and methods

Chromaffin granules were prepared from the adrenal medullae of freshly slaughtered cattle by homogenisation and differential centrifugation in 0.3 M sucrose containing 15 mM HEPES, pH adjusted to 6.6 with KOH [5]. Such preparations are more stable than those involving sucrose density gradient procedure [6]. Chromaffin granule membranes were prepared by successive dialysis of the chromaffin granule preparation against a buffer containing 15 mM HEPES, pH 6.6 as above, and initially 10 mM ascorbic acid and subsequent centrifugation. The experiments described below were all performed within 24 hr of the initial preparation of the granules.

Fluorescence measurements were made with an Hitachi Perkin-Elmer MPF-2A spectrofluorimeter equipped with a thermostatted cell block. The excitation wavelength was 380 nm and ANS fluorescence was followed at 480 nm. ATPase activities were determined using a Technicon Autoanalyser system which permits the continuous determination of the production of inorganic phosphate by a modified method of Hurst [7].

ATP was obtained from Boehringer and ANS from K and K Laboratories. ANS was recrystallised as the magnesium salt before use. All other reagents were of the highest purity commercially available.

3. Results and discussion

When equimolar quantities of $MgCl_2$ and ATP are added to a suspension of chromaffin granules in the presence of the fluorescent probe ANS there is a marked, time dependent enhancement of the probe fluorescence (fig.1). At pH 6.6 the half time for this increase is about 5 min. Addition of an uncoupler of mitochondrial oxidative phosphorylation causes this response to collapse with a half time of about 1.5 min. Addition of the uncoupler prior to that of Mg^{++} and ATP completely removes the fluorescence change.

Abbreviations: ANS: 1-anilinonaphthalene-8-sulphonic acid; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; S-13: 5-chloro,3-*t*-butyl,2'-chloro,4'-nitro-salycylanilide; FCCP: carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone; 1799: bis-hexafluoroacetone.

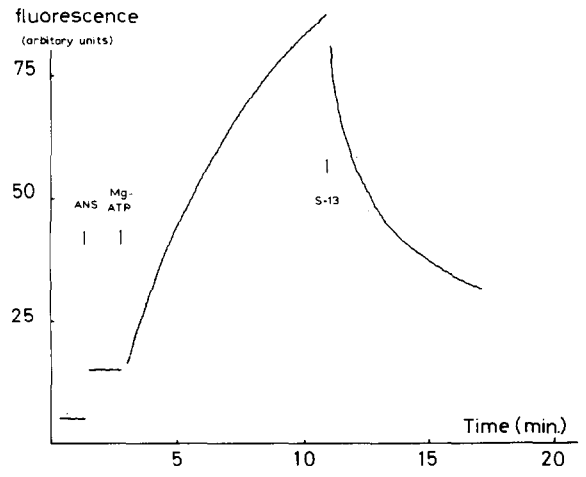

Fig. 1. The response of ANS to 'energisation' by Mg-ATP. ANS (5 μM) was added to chromaffin granules (0.25 mg protein/ml) in 0.3 M sucrose, 15 mM HEPES buffer pH 6.6. Mg-ATP (2.5 mM) was added to start the reaction. Uncoupling by 0.5 μM S-13.

The ATPase activity of the chromaffin granules under similar conditions is shown in table 1. At pH 6.6 the presence of uncoupler at low concentration (0.5 μM S-13) leads to a 4-fold increase in the ATPase activity. The effectiveness of different uncouplers at a given concentration (0.5 μM) decreases in the order S-13>FCCP>1799. The ionophore X-537A, which is reported to equilibrate both divalent metal ions and catecholamines across biological membranes [8], abolishes the ANS response to Mg-ATP 'energisation' and also enhances ATPase activity. Similar effects can also be induced by the addition of the permeant thiocyanate ion (final concentration 5 mM) and by making the membranes permeable to potassium by incubation in a buffer containing potassium (100 mM) and the ionophore valinomycin (1 μM) before conducting the experiment.

Table 1

Metal	Coupled	% Mg rate	Uncoupled	% Mg rate
Mg^{2+}	41	100	164	100
Mn^{2+}	40	98	146	89
Co^{2+}	37	90	108	66

Reactions were performed at 30°C in a buffer containing chromaffin granules (0.25 mg protein/ml), 0.3 M sucrose, 15 mM HEPES, 2.5 mM Mg-ATP pH 6.6. The reaction was started by the addition of the granules. The uncoupled rates were obtained in the presence of 0.5 μM S-13. The rates are in nmol of phosphate produced per minute per milligram of protein.

We have found no significant diminution of the ANS response to 'energisation' or of the ATPase activity of the chromaffin granules in the presence of a number of inhibitors of mitochondrial oxidative phosphorylation. Thus oligomycin (5 μM), aurovertin (2 μM) and atractylate (8 μM) all have no effect on the experiments described above. This is a clear indication that the responses we observe are not of mitochondrial origin but are due to the presence of the chromaffin granules. Experiments with the purified membranes of the chromaffin granules, which appear vesicular in electron micrographs, give similar results both with respect to the fluorescence responses and the ATPase activity. This indicates that the properties we observe are a function of the membrane portion of the chromaffin granules.

The dependence on pH of the ATPase activity, the ANS response and the response to uncoupling agent is shown in fig.2. Both the uncoupled ATPase activity and the response of the probe to 'energisation' have similar pH dependencies with optima at about pH 6.4. This also happens to be close to the pH optimum of the enzyme dopamine -β-hydroxylase (EC 1.14.2.1) which is an important component of the chromaffin granule membrane [9,10]. On the other hand the uncoupling of the ANS response by a given concentration of uncoupling agent (0.5 μM S-13) has a very different dependence. Below about pH 5.5 the effect of adding uncoupler is to further enhance the fluorescence of ANS. Below this pH the ATPase activity ceases to be uncoupler sensitive and indeed the activity may be inhibited by uncoupler. These observations suggest that if the role of the uncoupler is to equilibrate protons across the membrane then the pH of the chromaffin granule matrix is about pH 5.5.

When the system is tightly coupled the ATPase activity exhibits a very broad pH optimum, the activity being constant above pH 5.5. Furthermore in experiments at pH 6.6 use of other divalent metal ions than magnesium has little significant effect on the coupled ATPase rate (table 1). However, in the uncoupled system the different ions show differing

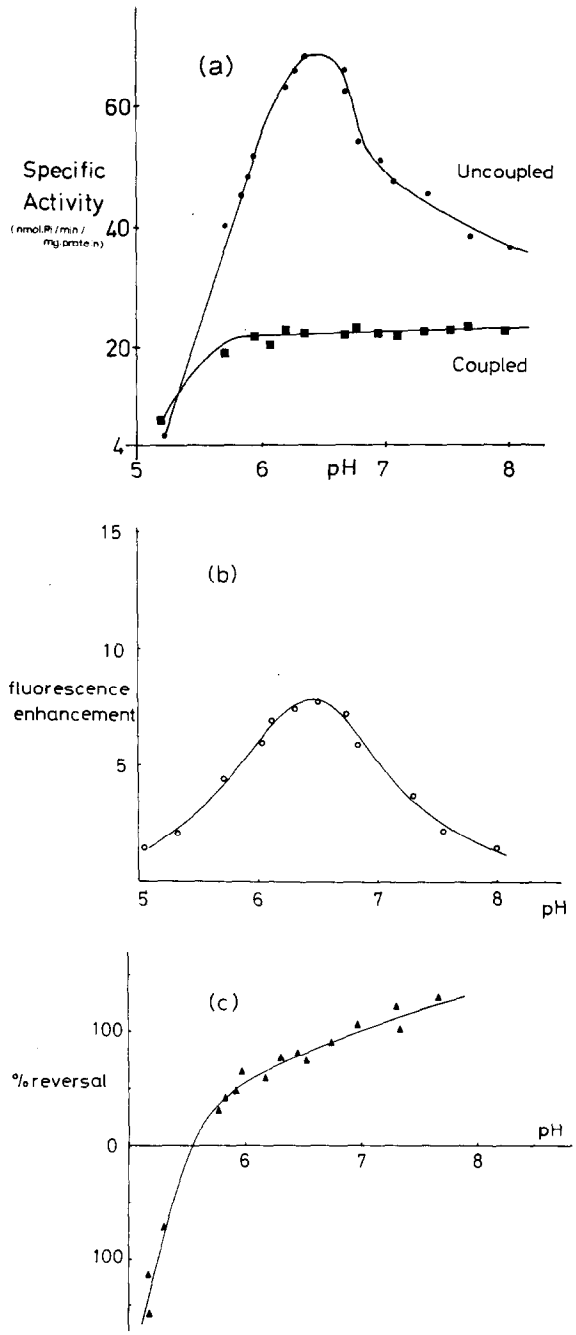

Fig. 2. The effect of pH on chromaffin granule (0.25 mg protein/ml) activities in 0.3 M sucrose, 15 mM HEPES buffer in the presence of 2.5 mM Mg-ATP. (a) ATPase activity in the presence (●—●—●) and absence (■—■—■) of 0.5 μM S-13. (b) Fluorescence enhancement of ANS (5 μM) bound to the granules. (c) The effect of 0.5 μM S-13 on the ANS response to 'energisation'.

efficiencies: $Mg^{2+}>Mn^{2+}>Co^{2+}$ (table 1). The ANS response to 'energisation' using magnesium or manganese is identical but is diminished with cobalt. Co^{2+}, however, is known as a good quencher of fluorescence. It seems, then, that in the coupled system the rate limiting step is not the turnover rate of ATPase activity and that this step is independent of both the pH of the buffer used and of the metal ion cofactor.

It is known that the 'energy-linked' ATPase activities of mitochondria, submitochondrial particles and the plasma membranes of some microorganisms are sensitive to uncoupling agents and inhibitors of oxidative phosphorylation. Changes in ANS fluorescence have also been correlated with 'energisation' in submitochondrial particles [11]. We believe this to be the first report of an uncoupler sensitive ATPase activity not of this general type. One interpretation of our observations is that the ATPase activity of chromaffin granule membranes is associated with the movement of cations, probably protons, across or into the membrane. Hence the sensitivity to uncoupling agents. Such a movement of protons would lead to the development of both electrical and chemical potential within or across the chromaffin granule membrane. The ANS response to 'energisation' may be to either or both of these components. However, the 'uncoupling' we observe under conditions where the membrane is leaky with respect to charged species (e.g. in the presence of permeant anion or of ionophores that move other cations than protons) seem to indicate that the element due to electrical potential is important both in terms of ATPase activity and in the ANS response to 'energisation'. Experiments with the purified chromaffin granule membranes, which retain these 'energy-linked' properties, are in progress. If the ATPase activity is indeed proton translocating then the active uptake of catecholamines may take place by a proton-catecholamine exchange mechanism.

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