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The assignment of the Ca^{2+}-ATPase activity of chromaffin granules to the proton translocating ATPase

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CaATP is shown to function as a substrate for the proton translocating ATPase of chromaffin granule ghosts at concentrations which are comparable to that of MgATP. Using the initial rate of the proton pump activity as the measure ($\Delta pH/\Delta t$), an apparent K_m-value of 139 ± 8 μM was estimated for CaATP and 59 ± 3 μM for MgATP. The maximal rate was markedly higher with MgATP than with CaATP, partly due to an inhibition of the hydrolytic activity at the higher concentrations of CaATP. The proton pump activity with CaATP was inhibited by *N*-ethylmaleimide and *N,N'*-dicyclohexylcarbodiimide at concentrations similar to that found for MgATP. No inhibition was observed with sodium vanadate in the concentration range 0–15 μM. Calmodulin and trifluoperazine had no effect on the overall ATPase activity with CaATP. These findings establish this acitivity as an intrinsic property of the chromaffin granules, i.e., linked to the H^+-ATPase. No evidence was obtained for the presence of a Ca^{2+}-translocating ATPase ($(Ca^{2+} + Mg^{2+})$-ATPase) in the chromaffin granules.

Ca^{2+}-ATPase *H^+-ATPase* *Proton pump* *Chromaffin granule* *Adrenal medulla*

1. INTRODUCTION

During the last twenty years a number of enzymatic activities, which result in a net hydrolysis of ATP, have been described in isolated chromaffin granules of the bovine adrenal medulla. A differentiation between the activities has been based partly on the dependency of the overall ATPase activity on the divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} [1–6] and partly on their coupling to transport of H^+ [7,8] and Ca^{2+} [9–12] or their function in various phosphorylation reactions, notably of phospholipids [4,6,13–15] and membrane proteins [4,6,15]. In addition to the intrinsic ATPase activity with MgATP as the substrate [1–3], which is to a large extent coupled to a vectorial transport of protons [7,8,16], particular interest has been focused on the Ca^{2+}-stimulated ATPase activity [3–5] and the proposed ATP-stimulated uptake of Ca^{2+} in intact chromaffin granules [9–12]. Previous measurements of the total ATPase activity in highly purified chromaffin granules have shown that CaATP gives 50% [3] or 20% [5] of the rate of hydrolysis measured with MgATP. However, it is not yet clear whether the ATPase activity with CaATP represents an intrinsic property of the chromaffin granules and is due to the same enzyme(s) as that/those using MgATP, or whether it is a different enzyme.

The purpose of this study is to provide evidence that CaATP indeed can substitute for MgATP as a substrate for the proton translocating ATPase in chromaffin granule ghosts as measured by the initial rate of proton pump activity.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; $(Ca^{2+} + Mg^{2+})$-ATPase, calcium-stimulated and magnesium-dependent ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; Pipes, piperazine-*N,N*-bis-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid

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2. MATERIALS AND METHODS

2.1. *Materials*

ATP (disodium salt, vanadium free), phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, calmodulin, dithiothreitol, *N*-ethylmaleimide and trifluoperazine were obtained from Sigma (MO, USA), DCCD from Koch-Light (Colnbrook, England), ANS from Eastman (NY, USA) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone from Boehringer (Mannheim, FRG). Sodium vanadate (ortho) was obtained from Fisher (NJ, USA). Essentially fatty acid free bovine serum albumin was obtained from Miles Biochemicals (IN, USA). The calcium ionophore A 23187 was a gift from Eli Lilly SA, USA.

2.2. *Preparation of chromaffin granule ghosts*

Highly purified resealed chromaffin granule ghosts were prepared from freshly collected bovine adrenal glands using sucrose gradient centrifugation as the final purification step [17,18]. 7.5 mM Mes buffer (pH 7.0) at 25°C, containing 0.1 mM dithiothreitol was used as the medium for lysis and washing of the chromaffin granules; the first washing also contained 0.2 mM EGTA and 0.1% (w/v) bovine serum albumin [18]. The crude ghosts were subjected to density gradient centrifugation to purify them from mitochondrial contamination [17,18]. The ghosts recovered from the gradient were stored in liquid nitrogen until used.

2.3. *Assay of ATPase activities*

The total Mg^{2+}-ATPase and Ca^{2+}-ATPase activities were assayed at 37 or 25°C in a medium containing 7.5 mM Pipes (pH 7.0), 100 mM NaCl, 1.25 mM CaATP or MgATP, 1 mM dithiothreitol, 2.5 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and 1 mM NH_4Cl. The formation of ADP was measured by high-performance liquid chromatography as described [19]. In these and other ATPase assays, conditions similar to that described by Pershadsing and McDonald [20] were used, including a calcium/EGTA or magnesium/EGTA buffer system to control the concentrations of free calcium or magnesium in the submicromolar and low micromolar range.

The rate by which the pH gradient (ΔpH) was generated across the chromaffin granule membrane was measured with acridine orange as a probe using an Aminco dual-wavelength spectrophotometer (Aminco, MA) set at 492 and 540 nm [18,21]. The partition of the weak base acridine orange across membrane vesicles has been found to be proportional to ΔpH, i.e. $[H^+]_i/[H^+]_o = [AO]_i/[AO]_o$, where the subscripts i and o refer to inside and outside of the ghosts, respectively [22]. The vectorial translocation of proton into the vesicles by an MgATP-driven proton pump is followed by movement of the unprotonated form of acridine orange into the vesicle, and when the protonated form accumulates on the inside, it aggregates and changes its absorption spectrum [22].

Changes in the transmembrane potential ($\Delta\psi$) were estimated from the changes in fluorescence of the probe ANS [23–25].

2.4. *Other analytical procedures*

Protein was determined [27] using bovine serum albumin as a standard.

The K_m-values for MeATP were estimated by non-linear regression analysis, using the programs described in [28], written in BASIC for the Apple IIe computer.

3. RESULTS

Intact chromaffin granules contain about 100 nmol $Ca^{2+} \cdot mg^{-1}$ protein and 20 nmol $Mg^{2+} \cdot mg^{-1}$ protein of which approx. 15% remains bound to the membrane when the granules lyse [29]. In order to circumvent this problem, the granule ghosts were washed with a medium containing EGTA in order to eliminate the possible contribution of externally bound Ca^{2+} and Mg^{2+} [30]. In addition, in certain experiments the incubation medium contained 200 μM EGTA in order to chelate any trace amounts of magnesium and calcium present in the reagents.

3.1. *Overall ATPase activity*

When the overall ATPase activity was assayed at pH 7.0 and 37°C, in the presence of a high concentration of ammonium chloride and a protonophore (FCCP), MgATP was found to give a 5-fold higher activity than CaATP. Typical values for the specific activities were 263 nmol $\cdot min^{-1} \cdot mg^{-1}$ protein and 53 nmol $\cdot min^{-1} \cdot mg^{-1}$ protein with 1.25 mM MgATP and 1.25 mM CaATP, respectively.

This relative number (i.e., 4.96) is in good agreement with that reported in [5].

Calmodulin (1 μg/ml) and trifluoperazine (30 μM) did not affect the overall ATPase activity with CaATP. Vanadate, in the concentration range 1–50 μM, inhibited in a similar way (i.e., by about 15% maximum) the overall ATPase activity with CaATP and MgATP as the substrate, with the same concentration dependency (not shown).

3.2. *Proton pump activity*

Acridine orange and ANS were used as probes to follow changes in ΔpH and $\Delta\psi$, respectively. As shown in fig.1a,b, a stable base-line was obtained within 5 min when EGTA-washed chromaffin granule ghosts were incubated in Pipes medium containing 50 mM KCl, acridine orange, and 200 μM EGTA, and 200 μM Mg^{2+} or Ca^{2+}. When 0.56 mM MgATP was added, the generated pH gradient (acidic inside) reached its half maximum value after about 55 s (fig.1a) and a maximum value after approx. 11 min. The addition of 0.48 mM CaATP also initiated the formation of a pH gradient (fig.1b) similar to that observed for 0.56 mM ATP, except that the initial rate and the maximal increase in the pH gradient was lower (see below), and the plateau was not reached within an 18 min reaction period. If NH_4Cl was added at this point, the pH gradient was immediately and completely dissipated, whereas the addition of FCCP caused a more gradual decay of the gradient (not shown), i.e., responses which were similar to that previously reported with MgATP [18].

As previously described, a similar response to MgATP was observed using the anionic fluorescent compound ANS as an extrinsic probe for the transmembrane potential (positive inside) [7,23] in a medium with a relatively impermeant anion (sulfate) [31]. CaATP was found to give a similar response as MgATP, but with a slightly higher apparent K_m-value for MeATP (see section 3.3).

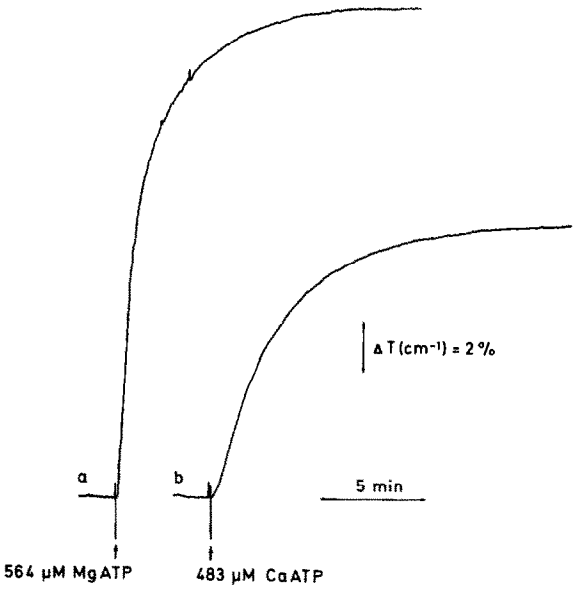

Fig.1. The time course for the change in absorbance of acridine orange induced by 0.564 mM MgATP (a) and 0.483 mM CaATP (b) in chromaffin granule ghosts. The change in $\Delta T_{492-540nm}$ of acridine orange in response to the generation of a pH gradient (acidic inside) at pH 7.0 and 25°C is as described in section 2. The reactions were initiated by the addition of MeATP; 98 μg granule protein · ml^{-1}.

3.3. *Effect of CaATP concentration on proton pump activity*

The relation between proton pump activity (i.e., energization) and the CaATP/MgATP concentration was determined by measuring the initial rate of the acridine orange absorbance change (fig.2) and ANS fluorescence change (not shown). The

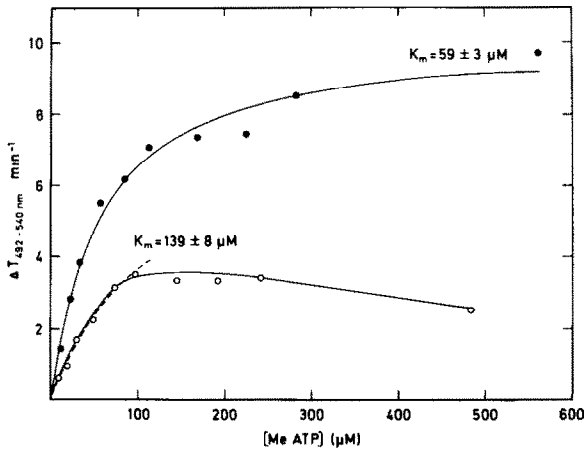

Fig.2. Concentration dependence of the proton pump activity in chromaffin granule ghosts with MgATP (●) and CaATP (○) as the substrate. The proton pump activity was measured as described in the legend to fig.1 and in section 2, and expressed as the initial increase in $\Delta T_{492-540nm}$ of acridine orange. The K_m-values given were determined by non-linear regression analysis [28].

apparent K_m-values were calculated by non-linear regression analysis [28] and found to be 59.1 ± 3.1 (mean ± SE) μM and 138.8 ± 8.4 (mean ± SE) μM for MgATP and CaATP, respectively (the acridine orange response). Using ANS as the probe the K_m-values were calculated to be 64.7 ± 7.7 μM for MgATP and 106.9 ± 11.6 μM for CaATP. The maximal rate with CaATP was not possible to estimate due to an inhibition at the higher concentrations of CaATP (fig.2).

Measuring the proton pump activity at 222 μM MgATP in the presence of a 200 μM Ca-EGTA buffer and a submicromolar concentration of free calcium, the simultaneous (to MgATP) addition of increasing concentrations of CaATP, in the range of 50–500 μM, resulted in a progressive inhibition of the initial rate of proton pump activity; at 500 μM CaATP the inhibition was about 40%.

3.4. *Effect of DCCD, NEM and vanadate*

Three compounds have so far been established as inhibitors of the proton translocating ATPase of chromaffin granules, i.e., DCCD [23,25,31], trimethyl tin [31] and *N*-ethylmaleimide (NEM) [23]. Here, DCCD and NEM were found to inhibit the proton pump activity in the same dose-dependent manner with CaATP as with MgATP as the substrate [23]; 50% inhibition was observed at 20.2 μM DCCD and 14.0 μM NEM.

It is particularly noteworthy that vanadate did not inhibit the proton pump activity with CaATP or with MgATP in the concentration range of 0–15 μM.

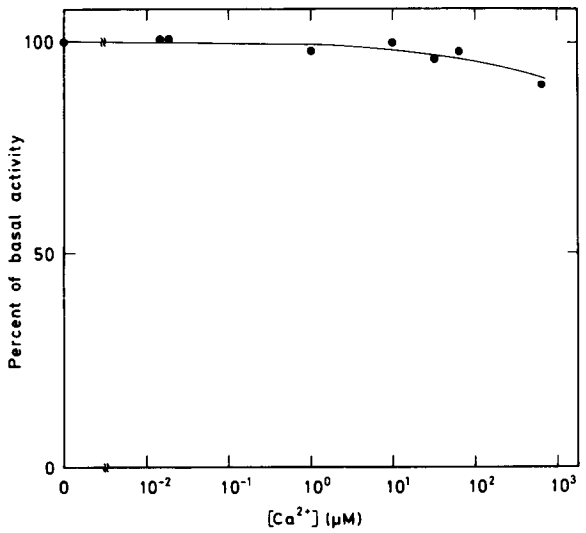

Fig.3. Effect of increasing concentrations of free calcium on the overall Mg^{2+}-ATPase activity of chromaffin granule ghosts. Standard assay conditions; all assays were conducted in the presence of 200 μM EGTA (see section 2). The basal Mg^{2+}-ATPase activity was 263 nmol·min^{-1}·mg^{-1} protein.

3.5. *Effect of free Ca^{2+} and calmodulin on the overall ATPase activity*

In the assay of the overall ATPase activity at 1.25 mM MgATP, the addition of increasing concentrations of free Ca^{2+}, in the range of 0.016–800 μM did not enhance the basal Mg^{2+}-ATPase activity (fig.3). On the contrary, a progressive inhibition was observed at increasing concentrations of added free Ca^{2+}. Furthermore, calmodulin (1 μg/ml) and trifluoperazine (30 μM) did not significantly affect the ATPase activity (not shown), and the Ca^{2+} ionophore A 23187 (20 μg/ml) slightly inhibited (by 14%) the activity.

4. DISCUSSION

A number of enzymatic activities, which result in a net hydrolysis of ATP, have been described in isolated chromaffin granules of the bovine adrenal medulla [1–8,13–16]. Among these activities a major fraction of the ATPase activity with MgATP as the substrate has been shown to represent an intrinsic property of the chromaffin granule membrane [1–3,7,8,16]. This conclusion is partly based on the high purity of the granule preparations obtained [1–3,8,16] and partly on the finding that the Mg^{2+}-ATPase activity is to a large extent coupled to a vectorial transport of protons [7,8,16,18,23, 31]. In addition, a Ca^{2+}-stimulated ATPase activity has also been reported to be present in this organelle [3–5], which is of particular interest in relation to the proposed ATP-stimulated uptake of Ca^{2+} in chromaffin granules [9–12].

Here, we have confirmed the presence of an ATPase activity with CaATP as the substrate [3–5] in highly purified chromaffin granule ghosts, with a maximal activity of about 20% of that measured with MgATP, in good agreement with reported values [5]. First, the ghosts were highly depleted of endogenous divalent cations by treatment with EGTA which effectively removes exter-

nally bound divalent metal ions [30]. Secondly, a low concentration of EGTA was included in the incubation medium to eliminate any possible contribution of the trace amounts of magnesium present in the assay medium. Thirdly, CaATP was found to inhibit the basal ATPase (fig.3) and proton pump activities with MgATP as the substrate. The degree of inhibition of the proton pump activity by increasing concentrations of CaATP, at fixed concentration of MgATP, was as expected for a competitive substrate with a lower affinity and a lower maximal rate.

When the ATPase activity was measured by the vectorial transport of protons, the maximal rate obtained with CaATP was found to be much lower than with MgATP (fig.2). Thus, the ATPase activity with CaATP in the chromaffin granule ghosts is fully accounted for by the intrinsic proton translocating ATPase, and the CaATP-driven proton pump shares all the basic properties of that driven by MgATP, including the dose-dependent inhibition by DCCD and NEM. The finding of a slightly higher K_m-value for CaATP than for MgATP was indeed expected from the rates obtained for overall ATPase activities with the two substrates, and it is similar to that recently observed for the ATPases in synaptosomes and synaptic membranes of rat brain tissue [32].

In addition to the electrogenic proton pump, the chromaffin granule membrane has been reported to contain an ATP-dependent mechanism of Ca^{2+} uptake [9–12] as well as a Na^+-Ca^{2+} exchange mechanism [30,33]. Although there seems to be agreement that the transport of calcium across the granule membrane is a carrier-mediated process [11,30,33,34], the energy source for the accumulation of calcium is highly controversial. Thus, it has been reported from several laboratories that neither ATP nor MgATP stimulate the uptake of Ca^{2+} by intact chromaffin granules [33,34] or by granule ghosts [30]. Indeed, ATP was found by these authors [30,33,34] to be somewhat inhibitory. On the other hand, experimental evidence has also been presented in support of an ATP-stimulated [9–11] or MgATP-stimulated [12] uptake of Ca^{2+}, and that this uptake is of high affinity. The mechanism of such a high-affinity uptake is not clear. The present study, however, does not support the presence of a Ca^{2+}-translocating ATPase in the chromaffin granules. Thus, as discussed above, the Ca^{2+}-ATPase activity is fully accounted for by the intrinsic H^+-ATPase. Furthermore, submicromolar and low micromolar concentrations of free calcium do not increase either the basal Mg^{2+}-ATPase activity (fig.3) or affect the positive membrane potential (not shown) induced by an optimal concentration of MgATP. In fact, CaATP as well as free calcium (fig.3) were found to inhibit the basal ATPase activity with MgATP, which is explained by the observation that CaATP functions as a competitive substrate for the proton translocating ATPase, and with a higher K_m-value than for MgATP. Finally, calmodulin and trifluoperazine had no effect on the Ca^{2+}-stimulated ATPase activity. Thus, all our data support the conclusion that the chromaffin granules do not contain a $(Ca^{2+} + Mg^{2+})$ATPase of the type found, e.g., in the microsomal fraction of bovine adrenal medulla [35] and rat brain synaptic membranes [32], and that the proposed MgATP-dependent uptake of Ca^{2+} in the chromaffin granules [12] must be explained by another mechanism.

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REFERENCES

[1] Hillarp, N.-Å. (1958) Acta Physiol. Scand. 43, 82–96.
[2] Kirshner, N., Kirshner, A.G. and Kamin, D.L. (1966) Biochim. Biophys. Acta 113, 332–335.
[3] Banks, P. (1965) Biochem. J. 95, 490–495.
[4] Trifaro, J.M. and Dworkind, J. (1971) Mol. Pharmacol. 8, 159–169.
[5] Trifaro, J.M., Duerr, A.C. and Pinto, J.E.B. (1976) Mol. Pharmacol. 12, 536–545.
[6] Muller, T.W. and Kirshner, N. (1975) J. Neurochem. 24, 1155–1161.
[7] Bashford, C.L., Casey, R.P., Radda, G.K. and Ritchie, G.A. (1975) Biochem. J. 148, 153–155.
[8] Flatmark, T. and Ingebretsen, O.C. (1977) FEBS Lett. 78, 53–56.

[9] Hausler, R., Burger, A. and Niedermaier, W. (1981) Naunyn-Schmiedebergs Arch. Pharmakol. 315, 255–267.
[10] Niedermaier, W. and Burger, A. (1981) Naunyn-Schmiedebergs Arch. Pharmakol. 316, 69–80.
[11] Burger, A. (1984) Trends Pharmacol. Sci. 5, 332–335.
[12] v. Grafenstein, H.R.K. and Neumann, E. (1983) Biochem. Biophys. Res. Commun. 117, 245–251.
[13] Trifaro, J.M. (1972) FEBS Lett. 23, 237–240.
[14] Phillips, J.H. (1973) Biochem. J. 136, 579–587.
[15] Buchley, J.T., Lefebvre, Y.A. and Hawthorne, J.N. (1971) Biochim. Biophys. Acta 239, 517–519.
[16] Njus, D. and Radda, G.K. (1977) Biochim. Biophys. Acta 463, 219–244.
[17] Terland, O. and Flatmark, T. (1980) Biochim. Biophys. Acta 597, 318–330.
[18] Husebye, E.S. and Flatmark, T. (1984) J. Biol. Chem., in press.
[19] Normann, P.T. and Flatmark, T. (1980) Biochim. Biophys. Acta 619, 1–10.
[20] Pershadsingh, H.A. and McDonald, J.M. (1980) J. Biol. Chem. 255, 4087–4093.
[21] Stone, D.K., Xie, X.-S. and Racker, E. (1983) J. Biol. Chem. 258, 4059–4062.
[22] Gluck, S., Kelly, S. and Al-Awgati, Q. (1982) J. Biol. Chem. 257, 9230–9233.
[23] Flatmark, T., Grønberg, M., Husebye, E. jr and Berge, S.V. (1982) FEBS Lett. 149, 71–74.
[24] Azzi, A. (1974) Methods Enzymol. 32, 234–246.
[25] Bashford, C.L., Casey, R.P., Radda, G.K. and Ritchie, G.A. (1976) Neuroscience 1, 399–412.
[26] Weber, G. and Yong, L.B. (1964) J. Biol. Chem. 239, 1415–1423.
[27] Bradford, M. (1975) Anal. Biochem. 72, 248–254.
[28] Knack, I. and Röhm, K.H. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1119–1130.
[29] Phillips, J.H., Allison, Y.P. and Morris, S.J. (1977) Neuroscience 2, 147–152.
[30] Phillips, J.H. (1981) Biochem. J. 200, 99–107.
[31] Johnson, R.G., Beers, M.F. and Scarpa, A. (1982) J. Biol. Chem. 257, 10701–10707.
[32] Michaelis, E.K., Michaelis, M.L., Chang, H.M. and Kitos, T.E. (1983) J. Biol. Chem. 258, 6101–6108.
[33] Krieger-Brauer, H. and Gratzl, M. (1982) Biochim. Biophys. Acta 681, 61–70.
[34] Kostron, H., Winkler, H., Geissler, D. and Konig, P. (1977) J. Neurochem. 28, 487–493.
[35] Goz, B. (1967) Biochem. Pharmacol. 16, 593–596.