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A Calcium Ion-Dependent Adenosine Triphosphate Pyrophosphohydrolase in Plasma Membrane from Rat Liver

DEMONSTRATION THAT THE ADENOSINE TRIPHOSPHATE ANALOGUES ADENOSINE 5′-[$\beta\gamma$-IMIDO]TRIPHOSPHATE AND ADENOSINE 5′-[$\beta\gamma$-METHYLENE]-TRIPHOSPHATE ARE SUBSTRATES FOR THE ENZYME

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An ATP pyrophosphohydrolase in a rat liver plasma-membrane subfraction was studied with respect to specific Ca^{2+} activation of the β-phosphate bond hydrolysis. ATP and, in addition, adenosine 5′-[$\beta\gamma$-imido]triphosphate and adenosine 5′-[$\beta\gamma$-methylene]triphosphate were substrates for Ca^{2+}-stimulated enzymic hydrolysis of the β-phosphate bond. A 15-fold activation was observed by raising the free Ca^{2+} concentration from 10^{-7} to 10^{-5}M. Mg^{2+} had little effect. Solubilization in 1% deoxycholate and partial purification on a sucrose density gradient resulted in a 5-fold increase in specific activity with unaltered Ca^{2+}-stimulation pattern. The possible importance of the enzyme in Ca^{2+} transport is discussed.

In 1967 Lieberman *et al.* reported the presence of an enzyme activity named nucleoside triphosphate pyrophosphohydrolase in rat liver plasma membranes. Very active ATP pyrophosphohydrolase activities associated with liver plasma membrane have later been observed by several authors (Ray *et al.*, 1970; Franklin & Trams, 1971; House *et al.*, 1972).

No function has yet been established for this enzyme in the membrane. Franklin & Trams (1971) found that the enzyme is not associated with acid–CoA ligase (EC 6.2.1.3) in plasma membranes.

It has previously been shown that ΔG for the hydrolysis of PP_i is near zero in the cell (Flodgaard & Fleron, 1974; Flodgaard, 1976). Consequently it can be deduced that ΔG for hydrolysis of ATP to AMP and PP_i in the cell is approx. 84kJ/mol (20kcal/mol) (Lawson *et al.*, 1976), and twice as large as ΔG for the hydrolysis of ATP to ADP and P_i. It is therefore tempting to associate the membrane-bound ATP pyrophosphohydrolase with reactions requiring more free-energy input than can be supplied by the hydrolysis of ATP to ADP and P_i. Extrusion of Ca^{2+} across the plasma membrane of the cell could be a candidate for such a reaction. In fact, on the assumption of an intracellular Ca^{2+} concentration of 10^{-7} to 10^{-8}M (Baker, 1976), an extracellular Ca^{2+} concentration of 10^{-3}M and a membrane potential of 70mV, the extrusion of 1mol of Ca^{2+} against the steady-state concentration and electrochemical gradients requires 38–42kJ (9–10kcal) [$\Delta G = RT\ln(c_{in}/c_{out})+|z|F\phi$]. This is equal to or very close to the free energy of hydrolysis of ATP to ADP and P_i. Thus, with a stoicheiometry of 1 Ca^{2+} ion transported to 1 ATP molecule hydrolysed to ADP and P_i, an efficiency of the pump near 100% is required.

Abbreviations used: p[N]ppA, adenosine [$\beta\gamma$-imido]-triphosphate; p[CH_2]ppA, adenosine [$\beta\gamma$-methylene]-triphosphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; ATPase, adenosine triphosphatase.

The object of the present investigation was to study the ATP pyrophosphohydrolase with respect to an activation by Ca^{2+}. To exclude interference by ATPases and adenylate kinase the enzyme was also studied by using ATP analogues not attacked by these enzymes (Yount *et al.*, 1971).

Experimental

Chemicals

[8-^{3}H]Adenosine 5′-[$\beta\gamma$-imido]triphosphate (ammonium salt; specific radioactivity 25Ci/mmol), [8-^{3}H]adenosine 5′-[$\beta\gamma$-methylene]triphosphate (ammonium salt; specific radioactivity 23Ci/mmol), 5′-[γ-^{32}P]ATP (sodium salt; specific radioactivity 3Ci/mmol), [^{3}H]AMP and [^{3}H]UTP were from The Radiochemical Centre, Amersham, Bucks., U.K. The purity of the ^{3}H-labelled ATP analogues was tested with respect to contamination with ATP by t.l.c. on poly(ethyleneimine)–cellulose in 2M-sodium for-

mate buffer, pH3.4. ATP contamination was less than 1%. Unlabelled ATP (disodium salt) and Tes were from Sigma Chemical Co., St. Louis, MO, U.S.A. Unlabelled p[NH]ppA, p[CH_2]ppA (both 98% pure, Boehringer certificate of analysis), AMP, UTP (sodium salts), adenosine, inorganic pyrophosphatase (EC 3.6.1.1), phosphoglucomutase (EC 2.7.5.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and UDP-glucose pyrophosphohydrolase (EC 2.7.7.9) were from Boehringer, Mannheim, Germany. Lumagel was from Lumac LSC Chemicals, Basel, Switzerland. Poly(ethyleneimine)–cellulose thin-layer sheets were product no. 5579 from Merck, Darmstadt, Germany. All other chemicals were of analytical grade.

Preparation of plasma membranes

House *et al.* (1972) described a procedure for the isolation of three subfractions of plasma membranes from rat liver. Only the low-density subfraction having a sucrose buoyant density of 1.13g/ml and being free of desmosomes, junctional complexes and bile canaliculi was used in the present investigation. Mitochondrial contamination as measured by monoamine oxidase activity with the method of Wurtman & Axelrod (1963) was found to be negligible, and the glucose 6-phosphatase activity (EC 3.1.3.9) measured by the method of Baginski *et al.* (1970) showed less than 10% contamination with microsomal particles.

Measurement of ATP pyrophosphohydrolase activity with ATP analogues as substrates

Incubation was carried out at 37°C in 20μl of the various buffers described in Table 1 containing ^{3}H-labelled analogue (2.5mM), diluted to a specific radioactivity of 2mCi/mmol after the removal of ethanol by freeze-drying. Then 1–10μg of protein was added and incubation carried out for 60min. After the end of the incubation 50nmol each of AMP and adenosine was added in a volume of 10μl. A 10μl portion was immediately spotted on poly(ethyleneimine)–cellulose thin-layer sheets, and the chromatogram was developed in 0.5M-LiCl for 10cm. The AMP+adenosine spot was cut out and extracted for 15min in 1ml of 4M-NH_3. The ^{3}H radioactivity was counted in 10ml of Lumagel in a Packard model 2425 Tri-Carb liquid-scintillation counter. The measured radioactivity was proportional to the amount of enzyme added in the assay and with incubation time for at least 90min. It was found that 100% of substrate could be hydrolysed to AMP+adenosine with excess of enzyme.

Measurements of ATP pyrophosphohydrolase activity in the solubilized subfraction freed from inorganic pyrophosphatase, and with ATP as substrate

The initial incubation procedure was the same as described above, except for the addition of [γ-^{32}P]ATP instead of the ^{3}H-labelled analogue. The reaction was stopped by boiling for 5min on a water bath. After cooling, 10nmol of carrier PP_i was added in a volume of 10μl. The [^{32}P]PP_i formed was incorporated into [^{3}H,^{32}P]UTP as described by Flodgaard & Fleron (1974). The nucleotides were separated by the method of Randerath & Randerath (1964), by using poly(ethyleneimine)–cellulose thin-layer sheets. The ATP and UTP spots were cut out and counted for radioactivity as described above; the latter was counted for both ^{3}H and ^{32}P radioactivity. From the ratio between the ^{3}H and ^{32}P radioactivities in the UTP spot the amount of PP_i produced from ATP was calculated. Enzyme activity was proportional to the amount of enzyme added, and with time for at least 1h.

Inorganic pyrophosphatase activity was measured under the same conditions as described above for the measurements of ATP pyrophosphohydrolase except for replacement of [γ-^{32}P]ATP with [^{32}P]PP_i at concentrations of 200, 100 and 10μM. After incubation for 1h with solubilized membranes residual [^{32}P]PP_i was measured as described.

Adenosine triphosphatase was measured as described by Formby *et al.* (1976).

Table 1. *Composition of Ca^{2+}- and Mg^{2+}-containing buffers*

The buffers were prepared in 20mM-Tes, pH8.0, containing 5mM-EDTA, 5mM-EGTA and 2.5mM of either ATP, p[N]ppA or p[CH_2]ppA. The total amounts of $CaCl_2$ and $MgCl_2$, to be added, to give the required free concentrations were calculated from the appropriate acid dissociation constants and complex-formation constants for all the components present in the buffers. The constants for ATP, EDTA, and EGTA were taken from Sillén (1971), and those for the two ATP analogues from Yount *et al.* (1971). The calculations were done on a Univac 1100 digital computer by using an ALGOL program described earlier (Flodgaard & Fleron, 1974). Ca^{2+} and Mg^{2+} binding to the various dissociated forms of the analogues is stronger than binding to ATP. The pK values for the terminal phosphate group in the analogues, however, is higher than for ATP, and thus replacement of ATP constants with analogue constants in the computer program did not alter the calculated total concentrations significantly.

Free concentrations (M)		Total concentrations (mM)	
Ca^{2+}	Mg^{2+}	$CaCl_2$	$MgCl_2$
10^{-3}	0	15.3	0
10^{-4}	0	11.9	0
10^{-5}	0	10.4	0
0	10^{-5}	0	5.38
10^{-5}	10^{-3}	6.83	6.62
10^{-6}	0	9.7	0
10^{-7}	0	7.8	0
10^{-8}	0	3.9	0

Protein was estimated by the method of Lowry *et al.* (1951) with the modifications of Lous *et al.* (1956).

Solubilization of the plasma membranes and partial purification of the ATP pyrophosphohydrolase

The plasma membranes were solubilized in 1% deoxycholate in 25mM-Tes, pH 7.5, for 1 h (0.75mg of protein/ml of solution) at 0°C. After dialysis overnight against excess of 25mM-Tes, pH 7.5, the solubilized membranes were placed on linear 3–30% sucrose gradients in a Beckman SW 27 rotor (0.75mg of protein per gradient) and centrifuged at 100000*g* for 24h at 4°C. The gradients were emptied through the top with a Beckman gradient fraction-recovery system, and 1 ml fractions were collected. The peak fractions containing most of the enzyme activity (15–21% sucrose) were pooled, dialysed and ultrafiltered against excess of 25mM-Tes, pH 7.5, to a final volume of about 125μl/0.75mg of membrane protein.

Results

A specific activity in the range 1.6–2.3μmol of AMP+adenosine/h per mg of protein was obtained with four individual preparations of plasma membrane subfraction with p[CH_2]ppA as substrate and a Ca^{2+} concentration of 10^{-5}M, with no Mg^{2+}, and at pH 8.0.

The membrane nature of the enzyme was checked by sonificating 330μg of membrane in 500μl of 50mM-Tes, pH 8.5, containing 1.0M-NaCl for 10s at 50W. Some heating was noticed, and enzymic activity fell to 63% of initial value ([Ca^{2+}] = 10^{-5}M, no Mg^{2+}, p[CH_2]ppA as substrate). After 1 h at room temperature (20°C) with occasional shaking, the preparation was centrifuged at 40000*g* for 15min at 4°C. All residual activity (47% of initial) was recovered in the pellet, although 20% of the initial protein was extracted in the supernatant. This result is in agreement with the findings of Lieberman *et al.* (1967).

A 15-fold stimulation of the specific activity of the solubilized enzyme with ATP as substrate is seen by raising the Ca^{2+} concentration from 10^{-7} to 10^{-5}M in the absence of Mg^{2+} (Fig. 1; for composition of buffers, see Table 1). A very small but reproducible (three experiments) stimulation is seen when the Ca^{2+} concentration is raised from 10^{-5} to 10^{-4}M. Another 2-fold increase in activity is observed by raising the Ca^{2+} concentration to 10^{-3}M (Fig. 1). The curves with p[N]ppA and p[CH_2]ppA as substrate show similar stimulation, but Ca^{2+} above 10^{-5}M inhibits. With p[CH_2]ppA as substrate, replacement of the maximum stimulatory Ca^{2+} concentration at 10^{-5}M with Mg^{2+} at 10^{-5}M and with no Ca^{2+} (all other conditions identical with those described in Fig. 1) results in near-zero activity (0.73μmol/h per mg of protein). At 10^{-5}M-Ca^{2+} and a physiological Mg^{2+} concentration (10^{-3}M), with

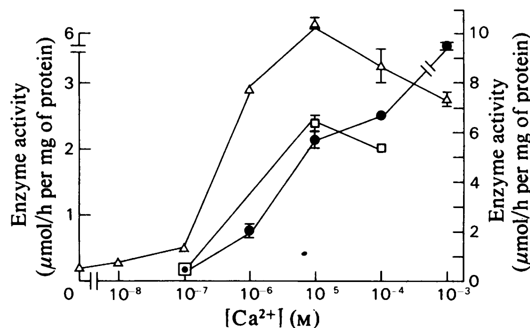

Fig. 1. *Specific activity of solubilized ATP pyrophosphohydrolase versus* [Ca^{2+}]

The assays were done at pH 8.0, 37°C for 60min in 20μl of 20mM-Tes buffer containing 2.5mM of either [^{32}P]ATP (●) (left ordinate), p[N]pp[^3H]A (□) (left ordinate) or p[CH_2]pp[^3H]A (△) (right ordinate), EDTA, EGTA (both at 5mM) and 1.2μg of solubilized enzyme. The total amounts of $CaCl_2$ added to give the Ca^{2+} concentration were calculated on a digital computer by using appropriate acid dissociation and complex-formation constants (see Table 1). [^{32}P]PP_i or [^3H]AMP+[^3H]adenosine were isolated at the end of incubation, and the specific radioactivities were calculated. The experiments were carried out in duplicate, and have been repeated at least three times with different enzyme concentrations. The Figure shows means of duplicate determinations in one experiment. Errors calculated from the duplicated determinations are indicated with bars when greater than the marks used.

p[CH_2]ppA as substrate a 25% decrease of the specific activity was observed compared with the same conditions without Mg^{2+}. Replacement of Ca^{2+} with Sr^{2+} or Ba^{2+} at free concentrations of 1.93×10^{-3}M and with no Mg^{2+} decreases the specific activity of the solubilized enzyme with p[CH_2]ppA as substrate at pH 8.0 to 14.0 and 15.4% of the activity obtained with 10^{-3}M-Ca^{2+} respectively. From the maximum activity of between 8.2 and 10.4μmol/h per mg of protein from three individual preparations of solubilized and purified enzyme with 10^{-5}M-Ca^{2+} with p[CH_2]ppA as substrate a 4.5–5-fold increase in specific activity by solubilization and partial purification can be deduced.

The solubilization rests on the criterion that no enzyme activity could be pelleted by centrifugation at 100000*g* for 1h at the end of the purification procedure (see the Experimental section). The preparation was completely free of inorganic pyrophosphatase (see the Experimental section), but some ATPase activity was still detectable in the presence of Mg^{2+}. However, in the absence of Mg^{2+} the only reactions taking place were the splitting of ATP to AMP+adenosine and PP_i, as the recovery of ^{32}P in the sum of [^{32}P]ATP and [^{32}P]PP_i was 100% (99.3±1.3%,

mean ± S.E.M., $n = 8$). The recovery of ^{3}H radioactivity in the sum of ^{3}H-labelled analogue and ^{3}H-labelled AMP+adenosine isolated from the assays was near 100% (for p[CH_2]ppA 97.4±1.1%, $n = 15$; for p[N]ppA 93.7±2.4%, $n = 10$), indicating selective hydrolysis at the β-phosphate position. p[CH_2)p and p[N]p were not isolated. Total recovery of enzyme activity after solubilization, gradient centrifugation, dialysis and ultrafiltration varied between 40 and 50% in three experiments, the greatest loss observed during the ultrafiltration and dialysis. The enzyme seems to be purified partly together with 5′-nucleotidase since a threefold increase in this activity in the final preparation was observed (10 μmol/h per mg of protein, measured at 30°C, pH 7.5 and with 0.1 mM-[^{3}H]AMP as substrate).

The high specific activity of the ATP pyrophosphohydrolase compared with the very low specific activity observed for adenylate cyclase in these membranes (House *et al.*, 1972) makes it unlikely that this enzyme should interfere. Measurement of ATP pyrophosphohydrolase in the presence of 1 mM-3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) showed negligible ^{3}H radioactivity in the cyclic AMP isolated.

Discussion

The present investigation has shown that an ATP pyrophosphohydrolase in rat liver plasma membranes was activated by Ca^{2+} in the physiological range of free concentrations from 10^{-8} to 10^{-4} M.

The results with the analogues of ATP are surprising. Although it has been shown that p[N]ppA can act as substrate in some reactions using the β-phosphate bond, breakdown of the much more unphysiological p[CH_2]ppA analogue at the β-position has never been reported before. Therefore the widely accepted inertness of this compound can no longer be sustained. In the present work it was shown to be even a better substrate than ATP (Fig. 1). The Ca^{2+}-stimulation pattern with this analogue supports the contention in the experiments with authentic ATP that it is the breakdown of the β-phosphate bond that is stimulated by Ca^{2+}.

Lieberman *et al.* (1967) measured the ATP pyrophosphohydrolase in plasma membranes at pH 12. They did not investigate Ca^{2+}-dependency, but observed a Mg^{2+}-dependence. They found a pH optimum at 10, and, since we found a higher specific activity at pH 9 compared with pH 7.5 (10.0 versus 6.22 μmol/h per mg of protein in one experiment with ATP as substrate), it is probably the same enzyme we have studied. The experimental conditions were, however, very different. Furthermore, the nearly zero activity at pH 7.5 observed by these authors may be caused by an Mg^{2+}-dependent inorganic pyrophosphatase. Our substantial activity observed at physiological pH with ATP as substrate was measured with a pyrophosphatase-free preparation.

The function of the enzyme is still unknown, and it is too early to conclude anything from the Ca^{2+} activation. The ability of the enzyme to break down ATP analogues may be of great help in evaluating the function, since ubiquitous inorganic pyrophosphatases and adenylate kinase will obscure the distinction between an ATPase and an ATP pyrophosphohydrolase with authentic ATP as substrate, but not with the analogues as substrates.

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