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KINETICS OF A NUCLEOTIDE PYROPHOSPHATASE IN THE PLASMA MEMBRANE OF THE FAT CELL

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Summary

Fat cells from rat and rabbit hydrolyzed externally applied adenosine triphosphate at a rate of about 1.8 nmol · mg^{-1} cells · min^{-1} corresponding to about 0.3 μmol · mg^{-1} protein · min^{-1}. Similar activities were found in cell homogenates. In purified adipocyte plasma membranes the rate of hydrolysis was about 1.8 μmol · mg^{-1} protein · min^{-1}. The hydrolytic activity was dependent on divalent metal ions. Mg^{2+}, Mn^{2+} and Ca^{2+} gave highest activities. The activity was maximal at about equimolar concentrations of M^{2+} and ATP. K_m for MgATP was about 0.23 mM and for CaATP about 0.36 mM. Combinations of Mg^{2+} and Ca^{2+}, or of Mg^{2+}, Na^+ and K^+ gave similar activities as did Mg^{2+} only. At concentrations of 1 mM the following nucleotides were hydrolyzed with a decreasing rate: ATP > ITP > GTP > UTP = CTP. In isolated fat cells the β-adrenergic drug isoproterenol and insulin slightly increased the rate of hydrolysis of external ATP, while the α-effector clonidine was inhibitory. The results suggest that a major portion of the ATP hydrolytic activity of the fat cell plasma membrane represents a nucleotide pyrophosphatase activity with access to externally applied ATP.

At the internal side of the plasma membrane of various types of cell there are several enzymes, e.g. ($Na^+ + K^+$)-ATPase, Ca^{2+}-ATPase and ($Mg^{2+} + Ca^{2+}$)-ATPase which transfer energy of the ATP molecule by hydrolysis into a vectorial transport of ions across the membrane [1,2]. In some cells, there is another group of enzymes in the plasma membrane which are capable of hydrolyzing nucleotide pyrophosphate bonds [3]. These enzymes have been

suggested to play a role in cell adhesion since their activity is absent in transformed cells in contrast with normal cells [3]. The activity of an externally localized adenosine triphosphatase was reported to be decreased in transformed cell lines [4].

The purpose of the present investigation was to characterize kinetically ATP-hydrolyzing enzymes in the adipocyte plasma membrane. This study shows that the adipocytes contain a nucleotide pyrophosphatase which requires Mg^{2+} or Ca^{2+}. This enzyme splits externally applied ATP and represents more than 90% of the total nucleotide hydrolyzing capability of the plasma membrane.

Experimental procedures

Materials. Nucleotides of highest purity, collagenase, trypsin, adrenaline and isoproterenol were obtained from Sigma. The Tris-salt of ATP was prepared by passing its sodium-salt over a Dowex 50W-X8 column in the Tris form [5]. Insulin was a product of Novo Industri AS (Copenhagen). Clonidine was a product of Boehringer Ingelheim. Other chemicals were of analytical grade and commerically available. Twice quartz-distilled, deionized water was used throughout the experiments.

Preparation of adipocytes and plasma membranes. Adipocytes were isolated routinely by collagenase treatment of epididymal and perirenal fat from 150—220 g rats or from rabbits [6]. In most experiments, when isolated cells were assayed for ATPase, rat adipose tissue was used. In order to obtain more easily relatively large amounts of plasma membranes, the rabbit adipose tissue was used. No kinetic difference in the ATPase of either the adipocytes or plasma membranes could be observed in these species. The nucleotide hydrolytic activity appeared to have similar substrate specificity and ionic requirements. Plasma membranes of collagenase-treated cells which had been washed twice in 0.25 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, were prepared by homogenization in 7 vols. of the same buffer [7]. The homogenate was centrifuged at 8000 × *g* for 5 min at room temperature. Floating fat was discarded. The following steps were performed at 0—4°C. The infranatant was centrifuged at 13 000 × *g* for 15 min. The pellet was resuspended in the original volume of buffer and the centrifugation was repeated at 13 000 × *g* for 15 min. The supernatants were combined and centrifuged at 35 000 × *g* for 2 h. The pellet was resuspended in the sucrose/Tris-HCl buffer at a protein concentration of about 2 mg/ml. This preparation was stored frozen at −25°C. The ATPase activity of the membranes was about 1—2 μmol · mg^{-1} protein · min^{-1}. The activity was stable for at least 2 months and the preparation could be freeze-thawed repeatedly without loss of activity.

ATPase assay. ATPase was assayed as the release of P_i according to Rathbun and Betlach [8]. Incubation volume for the activity assay of membranes was 500 μl. The standard assay medium contained 1 mM $MgCl_2$, 1 mM ATP in 20 mM Tris-HCl buffer, pH 8.0. After 10 min at 30°C, the reaction was interrupted by the addition of 15 μl 50% trichloroacetic acid. The color was developed as described [8].

In the standard ATPase assay of adipocytes, approx. 15 mg cells were

incubated with 1 mM ATP in 2 ml Krebs-Ringer/Hepes/albumin buffer, pH 7.4 [6]. The buffer contained 2% fraction V albumin. The original orthophosphate was excluded from this buffer. In some experiments this buffer was replaced by 0.3 M sucrose in 10 mM Tris-HCl buffer, pH 7.4. At indicated intervals of time, 250 μl of the assay mixture was transferred to a tube containing 300 μl 8% ice-cold trichloroacetic acid. The tubes were centrifuged in cold for 10 min at 1000 × *g*. P_i in the supernatant was assayed as described [8]. Appropriate blanks for cells, buffer and ATP were always included in the assay. The rate of hydrolysis was linear for at least 15 min.

Malate dehydrogenase assay. Malate dehydrogenase was assayed according to Ochoa [9].

Protein assay. Protein was determined according to Lowry et al. [10].

Results

ATPase activity in fat cells

The ATPase activity of intact adipocytes was compared with that of cell homogenates (Table I). Similar activities were observed. Fat cells were prepared normally by collagenase treatment of the adipose tissue. In order to test whether the collagenase had any effect on the ATPase activity, cells were prepared also by a nonenzymatic, mechanical procedure [7]. The ATPase activities in cells and in homogenates were about 2 nmol · mg^{-1} cells · min^{-1} irrespective of whether the cells were isolated by the collagenase method or by the mechanical procedure (Table I). Thus collagenase appeared not to change the enzyme activity.

The protein content of the cells was estimated after extraction of the lipids with chloroform/methanol [11]. About 0.6—0.8% by weight of the fat cell seemed to be protein. Thus the ATPase activity in the cells was about 0.3 μmol · mg^{-1} protein · min^{-1}.

Dependence on divalent cations of the ATPase of the adipocyte plasma membrane

In order to characterize kinetically the ATPase of the plasma membrane of the adipocyte, the ATPase activity of isolated membrane fragments was studied. The specificity for divalent cations was tested at concentrations of

TABLE I

COMPARISON OF THE ATPase ACTIVITY IN RAT ADIPOCYTES AND IN HOMOGENATES OF ADIPOCYTES

Adipocytes were prepared by the collagenase method [6] or by a mechanical procedure [7]. Intact cells or homogenates of cells were incubated at 30°C with 1 mM ATP, 1 mM $MgCl_2$ and 0.3 M sucrose in 10 mM Tris-HCl buffer, pH 7.4. Values are mean ± S.E.

Method	Activity (nmol · mg^{-1} cells · min^{-1})	
	Cells	Homogenate
Collagenase	1.93 ± 0.20	2.12 ± 0.30
Mechanical	1.99 ± 0.19	2.23 ± 0.15

0.2, 1.0 and 5.0 mM (Table II). Highest activity was observed at 1 mM Mg^{2+}. Mn^{2+} was a good substitute for Mg^{2+}. At 1 mM Mn^{2+}, about 88% of the activity with Mg^{2+} was obtained. At 0.2, as well as at 5 mM Mn^{2+}, the ATPase activity was reduced. Also, Ca^{2+} was a good substitute for Mg^{2+}. Maximal activity with 1 mM Ca^{2+} was about 80% of that with Mg^{2+}. With Ni^{2+} and Co^{2+}, only about 15 and 44%, respectively, of that with Mg^{2+} was obtained. Cu^{2+} was without effect.

In introductory experiments, the ATPase was assayed at various concentrations of Mg^{2+} at either 0.2 or at 1 mM ATP. In another set of experiments, the activity was assayed at various concentrations of ATP at either 0.2 or at 1 mM Mg^{2+}. In all experiments, maximal ATPase activities were observed at about equimolar concentrations of Mg^{2+} and ATP. In the absence of Mg^{2+} and other divalent metal ions, no activity was observed.

The ATPase activity was also tested with the concentrations of divalent cation and ATP held constant at a 1 : 1 ratio (Fig. 1). A K_m value for MgATP of about 0.23 mM was observed. V was about 1.8 $\mu mol \cdot mg^{-1}$ protein $\cdot min^{-1}$. When Ca^{2+} replaced Mg^{2+}, V was about the same, 1.7 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, but K_m for the Ca-ATP complex was 0.36 mM. In initial experiments, combinations of various cations were tested. Combinations of Mg^{2+} and Ca^{2+} were tested from 0.1 to 5 mM. No combination resulted in higher activity than with 1 mM Mg^{2+} only. On the other hand, when the total concentration of $Mg^{2+} + Ca^{2+}$ was increased to more than twice the concentration of ATP, a progressive inhibition was observed. Na^+ and K^+ in the incubation medium did not increase the ATPase activity significantly. The experimental error in the ATPase assay is about 5%. Thus at least 90% of the total ATPase of the fat cell membrane was distinct from $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase and $(Mg^{2+} + Ca^{2+})$-ATPase [1,2].

Dependence of the ATPase activity on the pH

The ATPase activity and stability was measured at various pH from pH 4 to 10 (Fig. 2). Optimal activity was at pH 8. A gradual decrease of the activity

TABLE II

ATPase ACTIVITY OF RABBIT ADIPOCYTE PLASMA MEMBRANES

Hydrolysis of ATP by plasma membranes of rabbit adipocytes in the presence of various divalent metal-ions at 30°C in the presence of 1 mM ATP and 20 mM Tris-HCl buffer, pH 7.4. The metal ions were added as their chloride salts except for Ni^{2+} and Cu^{2+} which were added as their acetate and sulfate salts, respectively. The activity obtained with 1 mM Mg^{2+} was the highest (1.5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) and represents the 100% value.

	Concentration of cation (mM)		
	0.2	1.0	5.0
Mg^{2+}	54	100	85
Mn^{2+}	57	88	53
Ca^{2+}	35	80	57
Co^{2+}	38	44	27
Ni^{2+}	9	9	15
Cu^{2+}	<5	<5	<5

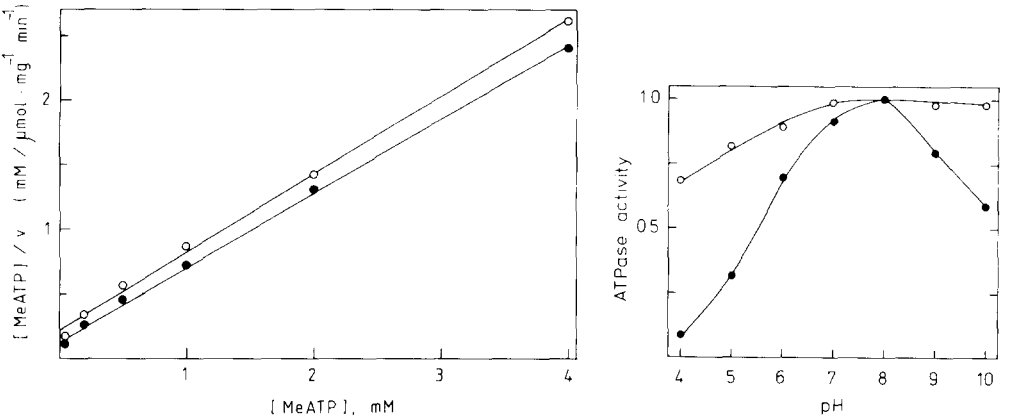

Fig. 1. Dependence on the rate of hydrolysis of ATP on the concentration of MATP (Hane's plot). Plasma membranes of rabbit adipocytes were incubated at 30°C with various concentrations of M^{2+} and ATP at a constant 1 : 1 ratio. Closed circles represent experiments with MgATP and open circles represent experiments with CaATP.

Fig. 2. Dependence of the ATPase activity on pH. Plasma membranes of rabbit adipocytes were incubated at 30°C for 10 min in the presence of 1 mM ATP and 1 mM $MgCl_2$ at various pH (•). Maximal activity was obtained at pH 8 and represents the 100% value. Stability tests of the ATPase were performed by incubation of the membranes at 30°C for 60 min at various pH. The pH was then adjusted to pH 8 and the activity of the ATPase was assayed (○). Buffers used were 3 mM acetic acid adjusted to pH 4.0 with imidazole; 3 mM morpholinosulfonic acid adjusted to pH 5.0 and to pH 6.0 with imidazole; 3 mM morpholinosulfonic acid adjusted to pH 7.0 with Tris; 3 mM Tris adjusted to pH 8.0, 9.0 and 10.0 with acetic acid.

was observed towards both extremes of pH. At pH 10, the ATPase activity was about 58% of maximal. At pH 4, the ATPase retained about 68% of its activity.

The stability of the enzyme was tested by incubating the enzyme for 1 h at 30°C and at various pH values. The pH was then adjusted to 8 and the activity was assayed. The enzyme was quite stable from about pH 7 to pH 10 (Fig. 2). Also, at acidic pH, the enzyme appeared to be rather stable. At pH 4, about 68% of the ATPase remained active.

Effect of inhibitors on the adipocyte plasma membrane ATPase

At 1 mM concentrations periodate, dinitrophenol and ouabain were without effect. Neither urea at 1 M concentration, nor 10 mM mercaptoethanol changed the enzyme activity. Half-maximal inhibition was obtained with 0.5 mM sodium azide/1 mM iodoacetic acid and with about 1.5 mM *N*-ethylmaleimide. The results indicate a role of a -SH group in the hydrolytic activity. When detergents were included in the incubation mixture low concentrations were already inhibitory. Half-maximal inhibition was obtained at about 0.003% Triton X-100 and at about 0.001% sodium dodecyl sulfate. Attempts to solubilize the enzyme in detergents invariably resulted in loss of all activity.

Substrate specificity

In order to test the substrate specificity of the enzyme, its activity towards various nucleotides was tested (Table III). At 1 mM concentration, ATP gave the highest activity. ITP and GTP were almost as effective—the activities were

TABLE III

SUBSTRATE SPECIFICITY OF ADIPOCYTE PLASMA MEMBRANE ATPase

Enzyme assays were performed at 30°C at 1 mM of indicated substrate, 1 mM $MgCl_2$ in the presence of 20 mM Tris-HCl buffer, pH 8.0. The activity with ATP was highest and represents the 100% value. *p*-NPP, *p*-nitrophenylphosphate.

Substrate	Relative activity
ATP	100
ITP	94
GTP	86
UTP	66
CTP	66
ADP	47
AMP	<5
p-NPP	0

94 and 86% of that with ATP. UTP and CTP both gave an activity of about 66% of that with ATP. ADP was split at a rate of about 47% of that of ATP. Only a very low activity was observed with AMP (<5%). *p*-Nitrophenylphosphate was not hydrolyzed.

In one experiment, the standard ATPase assay, which measures the liberation of P_i, was compared with the liberation of ADP by coupling the ATPase reaction to a pyruvate kinase assay using a large excess of pyruvate kinase [12]. Identical ATPase activities were obtained with the two methods. The present results, however, indicate that the hydrolytic activity represents a relatively unspecific nucleotide pyrophosphatase.

Effects of hormones

Adipocytes were incubated with various hormones for 10 min before the addition of Mg^{2+} and ATP (Table IV). Insulin as well as isoproterenol increased the hydrolysis of ATP by about 20—30% of the control, while the α-effector clonidine [13] was inhibitory. At 50 μM, adrenaline only slightly reduced the hydrolysis of ATP.

TABLE IV

Rat adipocytes were prepared by the collagenase method. The cells were washed three times with 0.3 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, and incubated for 10 min with various hormones. The ATPase activity of the cells was then assayed at 1 mM ATP, 1 mM $MgCl_2$ in 0.3 M sucrose and 10 mM Tris-HCl, pH 7.4. The activity without addition of hormone was taken as the 100% value. *n*, number of experiments.

Addition	Activity mean ± S.E. (*n*)
—	100.0 ± 7.9 (6)
Insulin 5 nM	122.4 ± 2.5 (6)
Isoproterenol 50 μM	130.2 ± 2.5 (6)
Adrenaline 50 mM	93.8 ± 3.7 (6)
Clonidine 100 μM	72.2 ± 11.5 (2)

Discussion

In a previous paper, Jarett and Smith demonstrated the existence of an insulin-stimulated Mg^{2+}-dependent ATPase in broken membranes of fat cells [14]. They did not, however, investigate the sidedness of the enzyme. From the activity data and the stimulation by insulin, it appears that the divalent metal-ion dependent nucleotide pyrophosphatase which is kinetically characterized in the present investigation is related to the previously described enzyme [14]. There are several indications that the hydrolytic site of the enzyme is in close association with the external surface of the cell membrane. In the ATPase assay, the cells did not contribute more than about 3% of the total absorbance. This low background allowed an accurate estimation of the ATPase in the complete incubation medium. Viability of the cells obtained with the collagenase procedure was controlled by measuring the activity of the intracellular enzyme malate dehydrogenase in the cell suspension at the end of each set of incubations. Homogenizing the cells resulted in a 5-fold increase of the malate dehydrogenase activity in the medium, while the ATPase activity essentially remained the same. Fat cells have been employed successfully in transport studies of sugar and cations [6,15]. There are no indications in these studies that the fat cell plasma membrane is freely permeable to externally applied ATP.

Enzymes which hydrolyze extracellular ATP were previously reported to be present in Ehrlich ascites tumor cells, glia cells, liver cells and lymphocytes [4,16,17]. Extracellular ATP increased the efflux of deoxyglucose, Rb^+, uridine and adenosine nucleotide pools in some transformed mouse cell lines [18]. In Ehrlich cells, the uptake of Ca^{2+} was increased by external ATP [19]. These results indicate that external ATP alters the permeability of the plasma membrane of various types of cell. The nucleotide hydrolyzing ability at the cell surface might therefore be connected with a permeability regulation mechanism. This idea is supported by the present finding that insulin and adrenergic effectors, which are all known to change the permeability characteristics to various solutes, altered the rate of hydrolysis of external ATP.

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