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DETECTION AND GENERAL PROPERTIES OF ADENOSINETRIPHOSPHATASE ACTIVITIES FROM PLASMA MEMBRANE IN RAT EPIDIDYMAL ADIPOSE TISSUE

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Abstract—A highly active Mg^{2+} or Ca^{2+} adenosinetriphosphatase activity has been detected in rat epididymal fat cell ghosts. ATP is not a specific substrate for the enzyme activity; ITP, UTP, CTP and GTP were in fact equally effective. The bivalent cation-dependent ATPase was significantly inhibited by oligomycin and by ethacrynic acid. 2,4-Dinitrophenol unaffected the reaction, whilst an ouabain inhibition was evident only in the presence of sodium azide. The ouabain sensitive ATPase activity corresponds to 38 per cent of the bivalent cation dependent ATPase. A sodium iodide purification procedure was then performed according to Nakao *et al.* as reported by Matsui and Schwartz. No (Na^+–K^+) ATPase activity was found in the purified enzyme, whereas a Mg^{2+} or Ca^{2+}-dependent activity was increased about 5-fold. The purified enzyme is more affected by oligomycin, whereas DCCD (*N,N'*-dicyclohexylcarbodiimide), an inhibitor of oxidative phosphorylation and of mitochondrial-stimulated ATPase activities is not active on the purified enzyme.

Data concerning the properties of the extracted enzymes and the further sodium iodide purification procedure are given.

RECENT studies on isolated fat cells[1,2] and on isolated fat cell "ghosts,"[3–7] have shown that the plasma membrane of rat epididymal adipose cells is the first site of action of different hormones and that several energy-requiring transport processes are operating at the same cellular level. An adenosinetriphosphatase activity was first observed by Kujalova and Mosinger[8] on membranes of fat cells. More recently the presence of a Mg^{2+}-dependent ATPase and of a Mg^{2+}-dependent (Na^+–K^+)-stimulated ATPase activity was evidentiated in the microsomal fraction of rat epididymal fat cells.[9] Finally, a highly active ATPase was detected by Rodbell *et al.*[5] in rat fat cell "ghosts."

This evidence strongly suggests that an appropriate ionic environment and an adequate energy availability could play an important role in controlling adipose tissue metabolism. In other words, it appears likely that also in adipose tissue the cellular sensitivity to different stimulus is mediated by a mechanism which correlates the energy availability and the ionic equilibrium at plasma membrane level.

In this paper a method is described for isolating and purifying the ATPase enzymatic systems operating in the plasma membrane of rat epididymal adipose tissue.

EXPERIMENTAL PROCEDURE

Isolation of plasma membrane. Fed male Wistar rats (200–250 g) were used. After light ether anaesthesia, rat epididymal fat pads (10–15 g) were excised, washed in

saline solution and immediately randomized and incubated for 60 min in Krebs–Ringer bicarbonate medium containing 2·5 per cent bovine albumin and collagenase. The fat cells were isolated according to Rodbell.[10] Sacs of intact plasma membrane, termed ghosts, were obtained from fat cell according to Rodbell[4] with the following modifications of the procedure.

Free fat cells were washed with 5 ml of cold hypotonic medium having the following composition: 2·5 mM $MgCl_2$, 0·1 mM $CaCl_2$, 1 mM $KHCO_3$, 2·5 mM ATP (disodium salt), 2 mM TRIS–HCl, at pH 7·4. The cell suspension was then centrifuged at 900 *g* for 15 sec at room temperature. The washing procedure was repeated in order to completely remove the Krebs–Ringer albumin medium and to obtain the swelling of the cells. Lysis was done as follows: the swollen fat cells were resuspended in 5 ml of the hypotonic medium and the suspension, after slow stirring, was centrifuged at 900 *g* for 5 min at room temperature.

At the end, the suspension resulted in three layers: a fat layer, a turbid aqueous phase and a small sediment. The aqueous-phase and the sediment representing the plasma membranes, were withdrawn and kept at 0°. The procedure on the fat layer was repeated four times so as to have a limpid aqueous phase without any sediment. The aqueous lysate collected after the four centrifugations was recentrifuged at 12,000 *g* for 15 min at 0°. The sediment was washed resuspending it in 5 ml of sucrose solution containing 0·25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 7·5 at 0° and then centrifuged at 12,000 *g* for 5 min at 0°. The washing procedure was repeated three times in order to completely remove ATP and Ca^{2+} from the medium. The final membrane pellet was resuspended in 2 ml of the sucrose solution and gently homogenized in a Potter–Elvehjem using a glass pestle, for 1 min at 0°. The homogenate, henceforth referred to as the plasma membrane fraction, was immediately used for the protein and the ATPase activity assays, or stored frozen at −28°.

The protein assays were performed on the homogenate by Lowry's method.[11]

ATPase activity determination. ATPase activity was determined as the rate of ATP hydrolysis during incubation of ATP with plasma membranes. The incubation was performed in a final volume of 1 ml containing about 50 μg of protein at 37° in a metabolic shaker for 20 min. Unless otherwise specified, the composition of the incubation medium was as follows: 3 mM ATP (disodium salt), 2·5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 50 mM TRIS–HCl, at pH 7·4. In the test, this solution will be referred to as the standard incubation medium.

The reaction was started after 5 min of preincubation at 37° by the addition of ATP to the test tubes, and stopped by adding 25 μl of 50 per cent trichloroacetic acid.

After a centrifugation at 12,000 *g* for 10 min at 0° the P_i release was determined by the Fiske–SubbaRow method,[12] and the ATPase activity was expressed in terms of specific activity (μmoles P_i released/mg protein/hr).

Sodium iodide purification of ATPase enzyme. The sodium iodide purification of ATPase enzyme was carried out according to Nakao *et al.*[13] as reported by Matsui and Schwartz.[14] The following modification of the procedure was made.

The plasma membrane suspension (3 mg protein/ml) was mixed with half of volume of 6 M NaJ solution containing 15 mM EDTA, 7·5 mM $MgCl_2$, 3 mM DTT (dithiothreitol = threo-1,4 dimercapto-2,3-butandiol) and 120 mM TRIS. The final pH of this solution was 8·5 at 0°. After gentle stirring, the mixture was kept for 15 min in an ice bath and then centrifuged at 105,536 *g* for 15 min at 0°. At the end, the mixture

resulted in three layers: a yellow supernatant film, a limpid aqueous phase and a small sediment. The film was resuspended in 2 ml of the following solution: 0·25 M sucrose, 10 mM TRIS, 1 mM EDTA, 1 mM DTT, pH 7·5 at 0°. After centrifugation at 105,536 *g* for 15 min at 0°, the sediment was resuspended in 2 ml of the same solution and re-centrifuged. The final sediment was gently homogenized in 0·2–0·3 ml of the same solution. This homogeneous suspension will be referred to as the sodium iodide purified enzyme. It was used immediately for the protein and the ATPase-activity assays, or stored frozen at −28° until used.

Reagents. All the organic and inorganic reagents; EDTA, ouabain, sodium azide and sodium fluoride were obtained from E. Merck (Darmstadt, Germany). Collagenase, ATP (disodium salt), ITP and UTP (trisodium salts), CTP and GTP (sodium salts), TRIS, bovine albumin fraction V, and DTT (dithiothreitol) were purchased from Sigma Chemicals (St. Louis, Mo., USA). Oligomycin (a mixture of oligomycin A and B) was supplied by the Upjohn Co. (Kalamazoo, USA). Rotenone, and 2,4-DNP were obtained from British Drug Houses Ltd. (Poole, England). DCCD (*N,N'*-dicyclohexylcarbodiimide) from Calbiochem (Los Angeles, USA). Ethacrinic acid from Merck Sharp & Dohme Res. Lab. (Rahway, N.J., USA).

RESULTS

Detection and general properties of ATPase activity in plasma membrane of rat epididymal adipose tissue

Bivalent cations dependency of ATPase activity in the crude preparation. Assays performed in plasma membrane fraction clearly establish the existence of a relatively high ATPase activity with an absolute requirement for bivalent cations Mg^{2+}, Ca^{2+} or Mn^{2+}. In their absence no ATPase activity was detected, even in the presence of Na^+ and K^+ (Table 1). Maximal specific activity was obtained in the presence of Ca^{2+}, or Mg^{2+} at a concentration 2·5 mM; Mn^{2+} 2·5 mM was less effective (60 per cent of the maximal activity) (Table 1). Moreover, the addition of Ca^{2+}, when Mg^{2+} was present at the optimal concentration, did not result in a further increase of ATPase activity. This strongly suggests that the enzymatic activity reaches its maximum in the presence of Ca^{2+} or of Mg^{2+}, 2·5 mM. This was more clearly evidentiated by following the ATP hydrolysis during the incubation time (Table 2). Results indicate that, with Mg^{2+} or Ca^{2+} 2·5 mM, with Ca^{2+} 1·25 mM + Mg^{2+} 1·25 mM, the activity reaches the maximum after 20 min and then decreases. However, when Ca^{2+} 2·5 mM is added to the incubation medium containing Mg^{2+} at the optimal concentration (2·5 mM) enzyme activity begins to decrease after the first 10 min of incubation.

Substrate specificity of ATPase. Table 3 shows that ATP is not a specific substrate for the ATPase activity. In fact, UTP, ITP, CTP and GTP can be utilized by the enzyme. However the highest activity was obtained in the presence of ATP 3 mM.

Effect of different inhibitors on ATPase. Various types of inhibitors and reagents that modify the ATPase(s) activity were tested on the crude preparation. 2,4-Dinitrophenol (10^{-5} and 10^{-4} M) did not affect the enzyme activity (Table 4). On the contrary ethacrynic acid and NaF strongly inhibited the ATPase activity (Table 3).

Table 5a presents the effect of oligomycin 10^{-5} M and of ouabain 0·1 mM. Oligomycin significantly inhibits the enzyme activity (about 35 per cent) whilst ouabain had no effect. Variations of K^+ or Na^+ concentration in the medium did not modify the

TABLE 1. BIVALENT CATION DEPENDENCY OF ATPase ACTIVITY

Bivalent cation in the medium (M)	Specific activity (μmoles P_i/mg protein/hr)
None	0
Mg^{2+} $0{\cdot}1 \times 10^{-3}$	0
Mg^{2+} $1{\cdot}0 \times 10^{-3}$	$61{\cdot}9 \pm 0{\cdot}92$
Mg^{2+} $2{\cdot}5 \times 10^{-3}$	$75{\cdot}1 \pm 0{\cdot}92$
Ca^{2+} $0{\cdot}1 \times 10^{-3}$	0
Ca^{2+} $1{\cdot}0 \times 10^{-3}$	$53{\cdot}8 \pm 0{\cdot}70$
Ca^{2+} $2{\cdot}5 \times 10^{-3}$	$61{\cdot}3 \pm 0{\cdot}74$
Mn^{2+} $0{\cdot}1 \times 10^{-3}$	0
Mn^{2+} $1{\cdot}0 \times 10^{-3}$	$42{\cdot}5 \pm 0{\cdot}80$
Mn^{2+} $2{\cdot}5 \times 10^{-3}$	$40{\cdot}7 \pm 4{\cdot}24$

After 5 min of preincubation at 37°, the reaction was started by adding ATP to the test tubes. The plasma membrane fraction (about 50 μg of protein) was incubated in a final volume of 1 ml at pH 7·5 in metabolic shaker at 37° for 20 min. The reaction was stopped by adding 25 μl of 50 per cent trichloroacetic acid and the inorganic phosphate was measured according to Fiske and SubbaRow method.[12] The composition of the standard incubation medium was as follows: 3 mM ATP (disodium salt), 2·5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 50 mM TRIS–HCl. Bivalent cation was completely removed from the medium or added at different molar concentration.

Each value is the mean ± S.E. of 19 different enzyme preparations.

TABLE 2. REPRESENTATIVE EXPERIMENT SHOWING THE EFFECT OF THE INCUBATION TIME ON ATPase ACTIVITY IN THE PRESENCE OF DIFFERENT BIVALENT CATION CONCENTRATIONS IN THE MEDIUM

Bivalent cation in the medium (M)	Incubation time		
	10 min	20 min	50 min
	(Specific activity: μmoles P_i/mg protein/hr)		
Mg^{2+} $2{\cdot}5 \times 10^{-3}$	35·0	39·2	29·8
Ca^{2+} $2{\cdot}5 \times 10^{-3}$	35·8	40·0	29·8
Mg^{2+} $1{\cdot}25 \times 10^{-3}$ + Ca^{2+} $1{\cdot}25 \times 10^{-3}$	38·8	39·2	28·6
Mg^{2+} $2{\cdot}5 \times 10^{-3}$ + Ca^{2+} $2{\cdot}5 \times 10^{-3}$	34·4	28·2	26·3

Experimental conditions as in Table 1. The reaction was stopped with 25 μl of 50 M trichloroacetic acid after 10, 20 or 50 min of incubation at 37° in a metabolic shaker.

TABLE 3. SUBSTRATE SPECIFICITY OF ATPase ACTIVITY IN THE PRESENCE OF Mg^{2+} OR Ca^{2+} IN THE MEDIUM

Substrate in the medium (M)		Mg^{2+} 2·5 mM (Specific activity: μmoles P_i/mg protein/hr)	Ca^{2+} 2·5 mM (Specific activity: μmoles P_i/mg protein/hr)
ATP	3×10^{-3}	63·2 ± 0·07	64·4 ± 1·70
UTP	3×10^{-3}	58·0 ± 3·16	63·2 ± 0·06
ITP	3×10^{-3}	56·1 ± 1·26	63·6 ± 1·06
GTP	3×10^{-3}	53·4 ± 1·36	51·4 ± 1·24 (P > 0·50)*
CTP	3×10^{-3}	52·3 ± 1·66	52·1 ± 6·10

Experimental conditions as in Table 1. ATP (disodium salt) ITP and UTP (trisodium salts), GTP and CTP (sodium salts) were dissolved in distilled water and the pH was adjusted at 7·4 with 0·1 M TRIS. Each value is the mean ± S.E. of three different enzyme preparations.

* P values are determined against the control assay having ATP in the medium.

response of crude enzyme to the glycoside. The experiments summarized in Table 5b, show that ouabain inhibits the ATPase activity only when sodium azide, a preferential inhibitor of Mg^2—ATPase[15–17] was present in the incubation medium. The ouabain-dependent ATPase activity corresponds only to 38 per cent of the total enzyme activity recovered in the plasma membrane fraction.

Detection and general properties of purified ATPase enzyme. In order to emphasize the presence of ouabain sensitive ATPase, the plasma membrane fraction was incubated according to Jorgensen and Skou,[18] in 1 ml containing 0·6 mg of sodium deoxycolate, 2 mM EDTA, 25 mM imidazole, pH 7·0 at 0°. After the incubation period of 30 min, the ATPase assay was performed as reported in the experimental procedure. No significant increase of the specific activity was obtained. On the contrary the ATPase activity was completely depressed.

TABLE 4. EFFECT OF METABOLIC INHIBITORS ON ATPase ACTIVITY

Drugs in the medium (M)	Specific activity (μmoles P_i/mg protein/hr*)	P†	% Inhibition‡
—	55·2 ± 7·80	—	—
2,4-DNP 10^{-5}	45·5 ± 2·29	> 0·30	—
2,4-DNP 10^{-4}	46·4 ± 0·57	> 0·30	—
Ethacrynic acid 5×10^{-3}	9·6 ± 4·8	< 0·05	83
NaF 2×10^{-2}	11·6 ± 2·90	< 0·05	79

Experimental conditions as in Table 1. The inhibitors were preincubated with the plasma membrane fraction (about 50 μg of protein) for 5 min at 37°. The reaction was started by the addition of ATP. The incubation was performed for 20 min in a metabolic shaker at 37°. The standard incubation medium was the same as in Table 1. Ethacrynic acid and sodium fluoride (NaF) were dissolved in distilled water. 2,4-Dinitrophenol (2,4-DNP) was dissolved in absolute ethanol. The concentration of ethanol introduced (10 μl/ml) did not affect the ATPase activity.

* Each value is the mean ± S.E. of three different enzyme preparations.

† P values and % inhibition (‡) are determined against the control.

TABLE 5. (a) EFFECT OF OUABAIN AND OLIGOMYCIN ON ATPase ACTIVITY; (b) EFFECT OF OUABAIN ON ATPase ACTIVITY IN THE PRESENCE OF SODIUM AZIDE (NaN_3)

	Drugs in the medium (M)	Specific activity (μmoles P_i/mg protein/hr)*	P†	% Inhibition ‡
a	—	61·0 ± 6·57	—	—
	Ouabain 10^{-4}	52·9 ± 4·35	> 0·30	—
	Oligomycin 10^{-5}	39·5 ± 6·50	= 0·05	35
b	—	43·8 ± 3·25	—	—
	NaN_3 10^{-2}	25·5 ± 2·80	< 0·001	42
	NaN_3 10^{-2} + Ouab. 10^{-4}	15·9 ± 4·08	= 0·05	38

Experimental conditions as in Table 4. Ouabain was dissolved in distilled water and oligomycin in absolute ethanol. The concentration of ethanol introduced (10 μl/ml) did not affect the ATPase activity.

* Each value is the mean ± S.E. of five to nine different enzyme preparations.

† P values and % inhibition (‡) are determined against the control.

In a further attempt to put in evidence a Na^+, K^+-activated ATPase, the crude enzyme was purified by sodium iodide[14] as described in the method. This procedure was in fact used by Matsui and Schwartz for the purification of a highly active ouabain sensitive Na^+, K^+-dependent adenosinetriphosphatase from cardiac tissue.[14]

After purification the ATPase activity has been increased approximately 5-fold in terms of specific activity (59·25 ± 5·7 μmoles P_i/mg protein/hr in the plasma membrane fraction; 254·6 ± 3·14 μmoles P_i/mg protein/hr in the purified enzyme). From the comparison between the crude and the purified enzyme, no differences were found in the Ca^{2+}, Mg^{2+} or Mn^{2+} cations requirement (Table 6), or in the inhibitory effect by oligomycin (—36 per cent) (Table 7).

However, it was interesting to find that the high specific activity of the purified enzyme was unaffected by ouabain even in the presence of 10 mM sodium azide (Table 7). These data clearly indicates that a highly active Mg^{2+}-dependent ATPase was obtained from plasma membrane of epididymal adipose cells and that the ouabain-sensitive component was lost during the purification procedure.

TABLE 6. BIVALENT CATION DEPENDENCY OF PURIFIED ATPase ACTIVITY

Bivalent cation in the medium (M)		Specific activity (μmoles P_i/mg protein/hr)*
None		—
Mg^{2+}	$2·5 \times 10^{-3}$	202·2 ± 3·43
Ca^{2+}	$2·5 \times 10^{-3}$	182·0 ± 3·76
Mn^{2+}	$2·5 \times 10^{-3}$	109·0 ± 2·75

Experimental conditions as in Table 1.

* Each value is the mean ± S.E. of three different enzyme preparations.

TABLE 7. EFFECT OF OUABAIN AND OLIGOMYCIN ON THE PURIFIED Mg^{2+}—ATPase ACTIVITY

Drugs in the medium (M)	Specific activity (μmoles P_i/mg protein/hr*)	P†	% Inhibition ‡
—	282 ± 11·10	—	—
Ouabain 10^{-4}	256 ± 16·18	> 0·30	9
Oligomycin 10^{-5}	181 ± 4·79	< 0·001	36
NaN_3 10^{-2}	147 ± 8·89	< 0·01	48
Ouabain 10^{-4} + NaN_3 10^{-2}	132 ± 9·05	> 0·30	—

Experimental conditions as in Table 4. Ouabain was dissolved in distilled water, oligomycin in absolute ethanol. The concentration of ethanol (10 μl/ml) did not affect the ATPase activity.

* Each value is the mean ± S.E. of three different enzyme preparations.

† P values and % inhibition (‡) are determined against the control.

Finally, in order to exclude the possibility of some mitochondrial contamination in the purified ATPase enzyme, the effect of DCCD (*N,N'*-dicyclohexylcarbodiimide) was investigated. The drug at concentrations of 10^{-7}, 10^{-6} and 10^{-5} M, had no effect on purified enzyme.

DISCUSSION

A highly active ATPase has been detected at the level of plasma membrane in the rat epididymal adipose tissue. The present results clearly indicate that this activity is Mg^{2+} and/or Ca^{2+} dependent. The specific activity of the ouabain-sensitive ATPase corresponds to 38 per cent of the Mg^{2+}-ATPase activity: these low (Na^+, K^+)-ATPase values could be explained taking into consideration that some important component could have disappeared together with some small fragment of the plasma membrane during the lytic process.[4] However, these low values are in agreement with the data of Modolell and Moore[9] showing the existence of a Mg^{2+} (Na^+, K^+)-activated ATPase system particularly concentrated in the microsomal subcellular fraction of rat epididymal adipose tissue. Taking into account the structure of adipose cells,[19] it seems possible that the ouabain sensitive ATPase may be only present in a small fraction of cytoplasmic membrane located near the nucleus and particularly rich in microsomes. On the contrary, the Mg^{2+}-activated ATPase may be equally present along the whole plasma membrane. This hypothesis could also justify the absence of the (Na^+, K^+)-ATPase and the presence of a very active Mg^{2+}-ATPase in the purified preparation. The lack of inhibition by DCCD, an inhibitor of oxidative phosphorylation[20,21] and only of mitochondrial dinitrophenol-stimulated ATPase activities, seems to indicate clearly that the activity of purified enzyme preparations are completely free from mitochondrial contaminations.

Moreover, it is interesting to remark that the action of oligomycin is not confined at mitochondrial level. In fact, like in other tissues[22,23] the drug shows an inhibitory effect also on plasma membrane ATPase activity. Whilst, on the contrary, DCCD, a drug that produces the same effect of oligomycin[20,21] on oxidative phosphorylation, has no effect.

Finally, it would be interesting to investigate the role of this bivalent cation dependent ATPase operating at the level of plasma membrane.

It has been observed that Ca^{2+} can be accumulated in mitochondria and in endoplasmic vesicles by energy requiring processes, and that in adipose tissue several conditions primarily affecting Na^+–K^+ transport or energy production are likely to change the concentration of Ca^{2+} in the cytoplasma.[24] This would suggest that in the plasma membrane of adipose cell, the active transport of bivalent and of monovalent cations could be in someway related. Thus, the Na^+–K^+ transport could control several metabolic pathways, since Ca^{2+} ions influence the activity of adenylcyclase and of other enzymes involved in lipolysis and glycogen metabolism.[24–30]

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