Quantitation and Characterization of the (Na\textsuperscript{+},K\textsuperscript{+})-Adenosine Triphosphatase in the Rat Adipocyte Plasma Membrane*

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The number of Na\textsuperscript{+} pumps in the rat adipocyte plasma membrane was determined by quantitating ouabain-dependent incorporation of inorganic \(^{32}\text{P}\) phosphate into the 95,000-dalton catalytic subunit of the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase. \(^{32}\text{P}\) incorporation was strictly dependent on the presence of ouabain, and was sensitive to Na\textsuperscript{+}, ATP, and alkali. There was no significant difference in the amount of ouabain-dependent phosphate incorporation and, therefore, in the number of active (Na\textsuperscript{+},K\textsuperscript{+})-ATPases between plasma membranes prepared from untreated or insulin-treated adipocytes; the maximal phosphorylation capacity was 10 pmol of phosphate/mg of membrane protein. There was no significant difference in the amount of \(^{3}H\)ouabain bound to plasma membranes from untreated or insulin-treated cells, and the number of binding sites with high affinity for ouabain (K\textsubscript{d} = 8 x 10\textsuperscript{-8} M) was 14 pmol/mg of membrane protein.

(Na\textsuperscript{+},K\textsuperscript{+})-ATPase activity was not significantly different in membranes prepared from control or insulin-treated cells, although only 10-20% of the ATP hydrolytic activity was ouabain-inhibitable. To obtain a more accurate estimation of (Na\textsuperscript{+},K\textsuperscript{+})-ATPase activity, hydrolysis of the pseudosubstrate \(\beta\)-(2-furyl)acryloyl phosphate (FAP), which was 60-70% ouabain-inhibitable, was monitored. Plasma membranes from control and insulin-treated cells exhibited nearly identical ouabain-sensitive FAPase activities. Low levels of ouabain-inhibitable FAPase activity were detected in the microsomal membrane fraction, and could be accounted for by plasma membrane contamination. It is concluded that the stimulatory effect of insulin on the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase in the intact cell is not retained through subsequent membrane isolation, and that the mechanism of insulin activation of the rat adipocyte (Na\textsuperscript{+},K\textsuperscript{+})-ATPase does not involve translocation of an intracellular pool of Na\textsuperscript{+} pumps to the cell surface.

The sodium and potassium ion-activated adenosine triphosphatase is the enzyme which mediates active transport of sodium and potassium ions across the plasma membrane of almost all eukaryotic cells (for review, see Ref. 1). There is increasing evidence that the intracellular concentrations of Na\textsuperscript{+} and K\textsuperscript{+} can be altered by the polypeptide hormone insulin (2-6). Several studies have shown that insulin effects on monovalent cation transport are blocked by cardiac glycosides (3-9), a class of specific inhibitors of the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase.\(^1\) Recently, it has been shown that in the hormone-responsive rat adipocyte, the uptake of Rb\textsuperscript{+} (a potassium analog) is rapidly and reversibly stimulated by insulin, and that this effect is a consequence of an insulin-induced activation of the adipocyte (Na\textsuperscript{+},K\textsuperscript{+})-ATPase (10). Kinetic analysis demonstrated that insulin acts by increasing the maximum velocity, \(V_{\text{max}}\), of Rb\textsuperscript{+} entry (10), a result which can be accounted for by an increase in the number of ion pumps or an activation of existing pumps. In addition, evidence has been presented that a strong correlation exists in the manner that both the adipocyte (Na\textsuperscript{+},K\textsuperscript{+})-ATPase and the transport system for glucose respond to insulin (10, 11).

A well documented primary event of insulin action is the stimulation of the uptake of glucose into responsive cells (12-14). It has been shown that this insulin effect is mediated by an increase in the \(V_{\text{max}}\) of the membrane-bound glucose transporter (12-14). Recently, methods have been developed to quantitate the number and activity of functional glucose transporters by inhibitor binding (15) and direct measurement of transport activity in a reconstituted system (16). Using these methods, Cushman and Wardzala (17), Wardzala and Jeanrenaud (18), Karnieli et al. (19), Suzuki and Kono (16), and Kono et al. (20) have presented evidence for an insulin-mediated translocation of glucose transporters from an intracellular microsomal pool to the plasma membrane. According to this mechanism, insulin enhancement of glucose transport activity is due to an increase in the number of transport molecules in the plasma membrane.

Based on the similarity in the insulin responsiveness of the fat cell transport systems for glucose and K\textsuperscript{+} (10, 11), it was of interest to determine if a change in the number of (Na\textsuperscript{+},K\textsuperscript{+})-ATPases in the plasma membrane could be detected following insulin treatment of adipocytes. Previous attempts to quantitate the number of ATPases have relied on measurements of binding of the specific inhibitor ouabain. However, there are inherent difficulties in interpreting these results due to the existence of nonspecific binding sites (5, 10), possible intracellular sequestration of ouabain (21), and binding to nonfunctional Na\textsuperscript{+} pumps (10). In this paper, a method is presented for quantitation of (Na\textsuperscript{+},K\textsuperscript{+})-ATPases that is stoichiometric with the number of active Na\textsuperscript{+} pumps. In addition, hydrolysis of a pseudosubstrate has been utilized to arrive at an accurate determination of (Na\textsuperscript{+},K\textsuperscript{+})-ATPase activity in the plasma membrane and microsomal membrane fractions of adipocytes. The studies reported in this paper demonstrate that functional (Na\textsuperscript{+},K\textsuperscript{+})-ATPases are present only in the plasma membrane, that insulin treatment of adipocytes does not result in a

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change in the number of plasma membrane Na⁺ pumps, nor is a permanent activation of (Na⁺,K⁺)-ATPase activity evident. These results imply that the Na⁺ pump responds only to signals effected by insulin in the intact cell, and suggest that, although a common signal may be generated by insulin binding to its cell surface receptor, the mechanism of insulin-induced increases in transport activities may not be the same for all transport proteins.

EXPERIMENTAL PROCEDURES

RESULTS

Quantitation of Na⁺ Pumps in the Adipocyte Plasma Membrane—The enzymatic mechanism of the Na⁺,K⁺)-ATPase involves phosphorylation of the 100,000-dalton catalytic subunit of the protein during the reaction sequence (1, 30). By quantitating the number of phosphorylated intermediates of the (Na⁺,K⁺)-ATPase, the number of active Na⁺ pumps can be determined. Using rat adipocyte plasma membranes and [γ⁻³²P]ATP as the phosphate donor, an initial attempt was made to identify a peptide of Mₙ = 100,000 whose phosphorylation was dependent on the presence of Na⁺ and Mg²⁺ and inhibited by K⁺ (1, 31). Analysis by polyacrylamide gel electrophoresis revealed a phosphorylated peptide of the expected molecular weight, but little or no effect by Na⁺ or K⁺ was observed. This peptide probably represented a phosphorylated intermediate of the Mg²⁺-ATPase (32), an enzyme whose activity comprised nearly 80–90% of the total adipocyte plasma membrane ATPase activity (see below).

An alternative procedure was employed in order to monitor phosphorylation specific only for (Na⁺,K⁺)-ATPase. In the presence of ouabain and Mg²⁺, an alkali-labile covalent phosphorylated intermediate of the enzyme can be formed from [³²P]phosphate with a stoichiometry of 1 mol of phosphate/mol of enzyme (33–35) which is chemically identical with that formed from [³²P]ATP + Mg²⁺ + Na⁺ (1, 35). Incubation of ouabain-treated rat adipocyte plasma membranes with [³²P]phosphate and subsequent analysis by acidic pH polyacrylamide gel electrophoresis revealed phosphate incorporation into 2 polypeptides migrating with Mₙ = 94,000 and 68,000 (Fig. 1). Phosphorylation of the 94,000-dalton peptide reached steady state levels in 10 min at 20 °C, with a half-time of approximately 30 s. If ouabain and [³²P]phosphate were added simultaneously to rat adipocyte plasma membranes, steady state phosphorylation required nearly 25 min at room temperature. This probably reflected the length of time required to achieve maximal ouabain binding. In the absence of ouabain, phosphorylation was 2% of the level reached in the presence of ouabain, and the addition of 100 mM Na⁺ resulted in an 84% inhibition of ouabain-dependent phosphorylation (Fig. 1). In contrast, phosphorylation of the 68,000-dalton peptide required almost 30 min at 20 °C to attain steady state levels, and was unaffected by the presence or absence of 1 mM ouabain (11) or 100 mM Na⁺ (11). Greater than 90% of the phosphate present in both bands was released when membranes were treated for 5 min with 2 mM ATP (Fig. 1) or when phosphorylated samples were analyzed at alkaline pH by the gel electrophoresis method of Laemmli (36) (data not shown). Based on these results, it is concluded that the 94,000-dalton phosphopeptide represents the phosphorylated intermediate of the rat adipocyte (Na⁺,K⁺)-ATPase. The identity of the 68,000-dalton peptide remains unknown, and the intensity of phosphorylation of this band varied among different membrane preparations. The insensitivity of phosphorylation to ouabain and Na⁺ makes it unlikely that this peptide is related to the (Na⁺,K⁺)-ATPase.

In order to assess the quantitative nature of the phosphorylation reaction, experiments were performed with purified dog kidney (Na⁺,K⁺)-ATPase (37). Although the amount of [³²P]phosphate in the band of Mₙ = 95,000 on an acidic pH gel was linearly related to the amount of protein applied to the gel, the yield of radioactivity from gel electrophoresis was less than 10% (data not shown). One possible explanation is that at an acid pH, a considerable amount of phosphate hydrolysis occurred during electrophoresis. Therefore, the amount of phosphate incorporation was determined directly on the trichloroacetic acid-precipitated and washed pellet using a phosphorylation procedure identical with that employed for rat adipocyte membranes. In the presence of 1 mM ouabain, phosphorylation to stoichiometric levels was achieved with purified (Na⁺,K⁺)-ATPase which exhibited Michaelis-Menten type dependence on phosphate concentration. The Kₘ of the purified enzyme for phosphate was 25 μM and maximal incorporation was 3.5 nmol of phosphate/mg. This represented 97%

2 Portions of this paper including “Experimental Procedures,” part of “Results,” Fig. 3, and Tables I and II are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1019, cite the author, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Rat adipocyte plasma membranes were phosphorylated at different concentrations of phosphate and the amount of ouabain-dependent phosphate incorporation into trichloroacetic acid-precipitated and washed membranes was quantitated. In a total of eight experiments of this type, the average maximal phosphorylation capacity was 9.96 pmol/mg (±1.7 pmol/mg, S.D.) with a \( K_m \) of 27 \( \mu \)M phosphate (±7 \( \mu \)M, S.D.). To determine whether insulin increased the number of (Na',K')-ATPases in the adipocyte plasma membrane, phosphorylation experiments were performed as described above on plasma membranes prepared from control and insulin-treated fat cells. The results of these experiments are depicted in Fig. 2 as a Lineweaver-Burk plot. No significant difference in ouabain-dependent phosphate incorporation was detected between control and insulin plasma membranes (\( p > 0.10 \), paired Student's \( t \) test). Similar results have been obtained in a total of three separate experiments on 3 different membrane preparations. These observations support the conclusion that insulin treatment of rat adipocytes does not alter the number of active (Na',K')-ATPase enzyme molecules in the plasma membrane.

The number of ouabain-dependent phosphorylation sites was also determined in the microsomal membrane fraction prepared from adipocytes. When analyzed in the manner depicted in Fig. 2 for plasma membranes, both control and insulin microsomes exhibited a maximal phosphorylation capacity of 0.6 pmol/mg of membrane protein (\( K_m \) = 35 \( \mu \)M phosphate). This was 7.6% of the value obtained for plasma membranes (see Fig. 2). Since the specific activity of 5'-nucleotidase in the microsomes was approximately 8% of that in the plasma membranes, and NADH-cytochrome c reductase activity was not significantly different between control and insulin microsome samples, it is concluded that the number of (Na',K')-ATPases detected in the microsomal membrane fraction can be accounted for by plasma membrane contamination.

The number of plasma membrane ouabain-binding sites did not change after insulin treatment of adipocytes. In addition, no significant difference in (Na',K')-ATPase activity was detected between plasma membranes prepared from control and insulin-treated fat cells. The results of these experiments are presented in the supplementary “Results” in the Mini-print. Taken together, these data imply that functional Na+ pumps are present only in the plasma membrane of the rat adipocyte and that stimulation of the (Na',K')-ATPase in the rat adipocyte does not involve translocation of Na+ pumps from an intracellular pool to the plasma membrane, nor is the stimulatory effect generated in the intact cell retained through subsequent membrane isolation.

**DISCUSSION**

In the present study, the number of (Na',K')-ATPase molecules in the rat adipocyte plasma membrane was determined by quantitating phosphorylated intermediate sites and ([H]ouabain-binding sites. Using both methods, approximately 6 \( \times 10^{12} \) Na+ pumps (10 pmol) were detected/mg of plasma membrane protein. This number was not significantly different in plasma membranes prepared from adipocytes which had been treated with insulin prior to cell disruption. There was no significant difference in (Na',K')-ATPase activity in plasma membranes prepared from control or insulin-treated cells determined from either ATP hydrolytic activity (which was only 15% ouabain-inhibitable) or by hydrolysis of the pseudosubstrate FAP, which was 60–70% inhibited by ouabain. Low levels of ouabain-sensitive FAPase activity, which were detected in the microsomal membrane fraction, could be accounted for by plasma membrane contamination. Based on these results, it is concluded that insulin stimulation of the (Na',K')-ATPase in rat adipocytes is not mediated by a change in the number of active Na+ pumps in the plasma membrane, and that the stimulatory effect is not retained through subsequent membrane isolation.

In this paper, a unique method has been utilized to measure the number of (Na',K')-ATPases. Formation of a phosphorylated intermediate from inorganic phosphate (rather than ATP) in the presence of ouabain virtually eliminates the contribution from other ATPases. Only active Na+ pumps should be detected, since the enzyme must turn over at least partially in order to incorporate phosphate (1). Since ouabain-dependent phosphorylation exhibits a Michaelis-Menten dependence on phosphate concentration, determination of \( ^{32} \)P incorporation at several phosphate concentrations allows extrapolation of the maximum phosphorylation capacity of the membranes. In principle, this method can be applied to measure the number of Na+ pumps in other cell types in which the
(Na',K')-ATPase comprises a small fraction of the membrane ATPase. In these experiments, variable amounts of an additional phosphopeptide of $M_r = 68,000$ were detected, a result which was also observed in phosphorylated plasma membranes from 3T3-L1 fibroblasts and adipocytes (11). Since no such phosphopeptide was observed when dog kidney microsomes or purified dog kidney (Na',K')-ATPase were phosphorylated, or when bovine serum albumin alone (without adipocyte membranes) was present in the reaction mixture (Fig. 1), it is unlikely that this peptide is simply phosphorylated albumin. Furthermore, the insensitivity of the 68,000-dalton phosphopeptide to ouabain and Na' make it improbable that it is related to the (Na',K')-ATPase and therefore, for the present time, the identity of this peptide remains unknown.

Repeated attempts to accurately measure (Na',K')-ATPase activity in adipocyte plasma membranes were frustrated by the presence of a large excess of Mg$^{2+}$ATPase activity, for which there is no known specific inhibitor. This problem was overcome by employing the pseudosubstrate assay described by Odom et al. (26). Under these conditions, it would have been possible to detect at least a 5% difference in ouabain-inhibitable FAPase activity between different membrane fractions. In addition, differences in membrane purity were corrected by normalizing FAP hydrolysis to the activity of 5'-nucleotidase, a specific plasma membrane marker in the adipocyte (17, 41). The results reported in this paper imply that active Na' pumps are present only in the rat adipocyte plasma membrane and that no difference in (Na',K')-ATPase activity is apparent when insulin is added either in vivo (to intact cells) or in vitro (to membrane fractions).

The existence of a Na' and K' ion-stimulated ATPase in rat adipocyte plasma membranes has been previously demonstrated by several investigators (24, 44, 45), all of whom noted the difficulty in accurately quantitating this enzyme. The activity and distribution of (Na',K')-ATPase reported in this paper are similar to those observed by others (24, 44, 45). However, the reported effects of insulin on ATPase activity are multifarious. Jaret and Smith (46) reported that direct addition of insulin to rat adipocyte plasma membranes caused a 12% increase in Mg$^{2+}$ATPase activity, with no change in the (Na',K')-ATPase, although Mg$^{2+}$ATPase activity was actually slightly lower in plasma membranes prepared from insulin-treated cells (46). Similarly, a 10-20% increase in apparent Mg$^{2+}$ATPase activity in response to direct addition of insulin to lymphocyte plasma membranes was reported by Hadden et al. (47). In contrast, incubation of rat muscle (48) or duck salt gland (6) with insulin prior to membrane isolation resulted in higher membrane (Na',K')-ATPase activity, whereas no effect could be observed when insulin was added directly to the membranes. Others have reported that direct addition of insulin to frog skeletal muscle stimulated (Na',K')-ATPase activity, but that this effect was only observed at submaximal concentrations of Na' and ATP (49). However, no alteration of (Na',K')-ATPase activity was detected in rat skeletal membranes (50) whether insulin was added directly to the membranes, or to intact muscle prior to homogenization, or was injected into rats prior to muscle isolation. The results described in this paper are in agreement with the latter report.

The possibility that insulin treatment alters the affinity of the (Na',K')-ATPase for Na' (3, 49) cannot be ruled out. However, since only 15% of the ATPase activity is ouabain-inhibitable (and therefore Na'-dependent) and hydrolysis of FAP does not require Na', it would be extremely difficult to ascertain whether an alteration of the $K_m$ for Na' occurred in the rat adipocyte (Na',K')-ATPase.
tation with the insulin receptor, the actual mechanism mediating the increase in transport activity of the Na\(^+\) pump and the glucose transporter is apparently different.

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REFERENCES

Rat Adipocyte Plasma Membrane (Na⁺,K⁺)-ATPase

Marilyn G. Rein

SUPPLEMENTAL MATERIAL

QUANTITATION AND CHARACTERIZATION OF THE 
(Na⁺,K⁺)-ATPASE TRYP SINHYDRAZINe IN THE RAT ADIPOCYTE PLASMA MEMBRANE

EXPERIMENTAL PROCEDURES

Preparation of Rat Adipocyte Membrane Fractions

Adipocytes were homogenized as described previously (18). Washed cells were made up to a 30% packed cell volume in Krebs-Ringer phosphate buffer containing 1% bovine serum albumin. Cell suspensions were homogenized in 125 ml polypropylene separatory funnels (Nalgene) at 37°C for 5 min, followed by centrifugation in the presence of digitonin (16-20,000×g) for 15 min in an IEC 20 model ultracentrifuge. The supernatant was decanted, and homogenization buffer (35 ml) sucrose, 20 mM Tris-Cl, pH 7.4, with or without digitonin, was added to a final volume of 55 ml.

Preparation of rat adipocytes was accomplished as described by Cashman and Naldini (17). The cell suspension was divided into a 5 ml portion-Evans blue dye (Hoechst) and homogenized at 3°C, then added to 30 ml of 16,000×g homogenization buffer, followed by the addition of digitonin. The homogenate was centrifuged at 100,000×g for 1.5 h at 4°C. The supernatant was decanted, and homogenization buffer (25 ml) sucrose, 20 mM Tris-Cl, pH 7.4, with or without digitonin, was added to a final volume of 55 ml.

Washed rat adipocytes were prepared by the method of Mefford and Jaffee (12) as described by Cashman and Naldini (17). The cell suspension was divided into a 5 ml portion-Evans blue dye (Hoechst) and homogenized at 3°C, then added to 30 ml of 16,000×g homogenization buffer, followed by the addition of digitonin. The homogenate was centrifuged at 100,000×g for 1.5 h at 4°C. The supernatant was decanted, and homogenization buffer (25 ml) sucrose, 20 mM Tris-Cl, pH 7.4, with or without digitonin, was added to a final volume of 55 ml.

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Rat Adipocyte Plasma Membrane (Na⁺,K⁺)-ATPase

**Material**

Plasma membrane ATPase activity was measured to determine whether increased Na⁺ pump activity was retained following disruption of insulin-treated cells. The results of separate experiments from eight different, paired (control and insulin-treated) plasma membrane preparations are presented in Table I. Similar results have been obtained in a total of 6 experiments. No significant difference (p>0.05) was detected in total (Table I) or ouabain-resistant Na⁺ pump activity (Table II) in plasma membranes from control and insulin-treated adipocytes. No significant difference in FAPase activity was detected in plasma membranes from control and insulin-treated cells. Plasma membranes from control and insulin-treated adipocytes were prepared as described in the Experimental Procedures. The results were calculated as the difference between control and insulin-treatment (p<0.05).

**Table I**

<table>
<thead>
<tr>
<th>Plasma Membrane ATPase Activity</th>
<th>µmol/mg/min</th>
<th>Na⁺,K⁺-ATPase activity</th>
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<tbody>
<tr>
<td>Control</td>
<td>86</td>
<td>145</td>
</tr>
<tr>
<td>Insulin</td>
<td>73</td>
<td>33</td>
</tr>
<tr>
<td>Difference: Control vs. Insulin</td>
<td>N.S. p&gt;0.10</td>
<td>N.S. p&gt;0.10</td>
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</tbody>
</table>

**Table II**

<table>
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<tr>
<th>Adipocyte Membrane FAPase Activity</th>
<th>µmol/mg/min</th>
<th>Na⁺,K⁺-ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>77.7</td>
<td>48.2</td>
</tr>
<tr>
<td>Ouabain</td>
<td>28.5</td>
<td>41.7</td>
</tr>
<tr>
<td>Difference: Total vs. Ouabain</td>
<td>N.S. p&gt;0.10</td>
<td>N.S. p&gt;0.10</td>
</tr>
</tbody>
</table>

**Results**

Plasma membranes from untreated (control) and insulin-treated (insulin) adipocytes were preincubated at 25°C for 20 min in the absence (total) or presence (ouabain) of 10 µM ouabain and ATPase activity was monitored at 37°C as described in the Experimental Procedures. Data are presented in Table II. The Na⁺,K⁺-ATPase activity was calculated by subtracting the ouabain-resistant activity from the total activity. There was no significant difference in NADH-cytochrome c reductase activities between control and insulin-treated samples (data not shown). No statistically significant difference (p<0.05) was detected in the Ouabain-sensitive FAPase activity in plasma membranes from control and insulin-treated cells. The results of separate experiments from eight different, paired (control and insulin-treated) plasma membrane preparations are presented in Table I. Similar results have been obtained in a total of 6 experiments. No significant difference (p>0.05) was detected in total or ouabain-resistant Na⁺ pump activity (Table II) in plasma membranes from control and insulin-treated adipocytes. No significant difference in FAPase activity was detected in plasma membranes from control and insulin-treated cells.

**Discussion**

The results of separate experiments from eight different, paired (control and insulin-treated) plasma membrane preparations are presented in Table I. Similar results have been obtained in a total of 6 experiments. No significant difference (p>0.05) was detected in total or ouabain-resistant Na⁺ pump activity (Table II) in plasma membranes from control and insulin-treated adipocytes. No significant difference in FAPase activity was detected in plasma membranes from control and insulin-treated cells. Plasma membranes from control and insulin-treated adipocytes were prepared as described in the Experimental Procedures. The results were calculated as the difference between control and insulin-treatment (p<0.05).

**References**

1. M.D. Resh, unpublished observations.