Characterization of the Adipocyte Ghost (Na\(^+\),K\(^+\)) Pump

INSIGHTS INTO THE INSULIN REGULATION OF THE ADIPOCYTE (Na\(^+\),K\(^+\)) PUMP*

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The (Na\(^+\),K\(^+\)) ATPase in plasma membranes isolated from rat adipocytes is insensitive to insulin (Lyttón J., Lin, J. C., and Guidotti, G. (1985) J. Biol. Chem. 260, 1177-1184). For this reason, the characteristics of the (Na\(^+\),K\(^+\)) pump in adipocyte ghosts, prepared by hypotonic lysis of adipocytes (Rodbell, M. (1967) J. Biol. Chem. 242, 5744-5754), were studied. Herein it is demonstrated that the (Na\(^+\),K\(^+\)) pump in ghosts is identical to that described in isolated plasma membranes, sharing the following characteristics: 1) the K\(_{\text{a}}\) values for ouabain are 1.3 \(\times 10^{-7}\) M and 4.5 \(\times 10^{-5}\) M for the \(a2\) and \(a1\) isozymes, respectively; 2) the K\(_{\text{a}}\) values for sodium are 11.4 \(\pm 1.6\) and 7.2 \(\pm 3.8\) mM for the \(a2\) and \(a1\) isozymes, respectively; 3) both forms of the (Na\(^+\),K\(^+\)) pump are insensitive to insulin stimulation, presumably because the activities are already maximal. The ghosts are not in an insulin-stimulated state because the activity of the glucose transporter is not increased as it is in ghosts prepared from insulin-treated cells.

In addition, presented evidence demonstrates that ghost internal sodium concentration, [Na\(^+\)], is very sensitive to changes in the activity of the (Na\(^+\),K\(^+\)) pump. If the [Na\(^+\)] of adipocytes is very sensitive to the activity of the (Na\(^+\),K\(^+\)) pump, the mechanism of insulin stimulation of the adipocyte (Na\(^+\),K\(^+\)) pump requires reexamination.

The (Na\(^+\),K\(^+\)) ATPase is the enzyme responsible for maintaining potassium and sodium gradients across the plasma membrane of almost every eukaryotic cell (for reviews see Cantley, 1981, 1986; Forgac and Chin, 1985; Jorgensen and Andersen, 1988). The basic unit of the enzyme is an \(a\) dimer, with \(a\) the polypeptide acting as the catalytic subunit. There are three isoforms of the \(a\) chain: \(a1\), \(a2\), and \(a3\) (Sweedner, 1979; Hsu and Guidotti, 1988; Shull et al., 1986). (Before cDNAs for each subunit were cloned and sequenced, \(a\) referred to the kidney isoform (\(a1\)) and \(a+\) referred to the brain isoform(s) (\(a2\) or \(a2/a3\)) of the \(a\) subunit.)

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‡ The abbreviations used are: (Na\(^+\),K\(^+\))-ATPase or (Na\(^+\),K\(^+\)) pump, sodium and potassium ion-activated ATP phosphohydrolase; BSA, bovine serum albumin; KRH, Krebs-Ringer-HEPES; K\(_{\text{a,Na}}\), the concentration of Na\(^+\) which gives half-maximal stimulation of the (Na\(^+\),K\(^+\)) pump; [Na\(^+\)], and [Na\(^-\)], internal and external Na\(^+\) concentrations, respectively.

The activity of the (Na\(^+\),K\(^+\)) pump can be regulated by hormones (Rossier et al., 1987). For example, in some cells, insulin stimulates the activity of the (Na\(^+\),K\(^+\)) pump by increasing the intracellular Na\(^+\) concentration (Fehlmann and Freychet, 1981; Rosic et al., 1985). In skeletal muscle and adipocytes, insulin does not increase the intracellular Na\(^+\) concentration (Clausen and Hansen, 1977; Clausen and Kohn, 1977; Resh et al., 1980; Lyttón et al., 1985; our own results, data not shown); the Na\(^+\) affinity of the \(a2\) isoform of the pump appears to be affected (Lyttón, 1985). The \(a1\) isozyme in adipocytes has a high Na\(^+\) affinity, and its activity is virtually unaffected by insulin. In plasma membranes prepared from adipocytes, both forms of the (Na\(^+\),K\(^+\)) ATPase have high Na\(^+\) affinities and their activities cannot be stimulated by insulin (Lyttón et al., 1985). With the exception of one report (Gavryck et al., 1975), insulin only stimulates the (Na\(^+\),K\(^+\)) pump in whole cells. These data have led to the hypothesis that insulin may stimulate the activity of the \(a2\) form of the pump by partially releasing an inhibition that decreases the \(a2\) isozyme’s Na\(^+\) affinity in cells (Lyttón, 1985). Upon preparation of membranes, the putative inhibitory molecule could be lost or inactivated, allowing the \(a2\) isozyme to exist in a high Na\(^+\) affinity state similar to that of the \(a1\) isozyme.

The glucose transporter is another adipocyte membrane protein whose activity can be stimulated by insulin (for reviews, see Kono, 1983; Simpson and Cashman, 1986). Unlike the stimulation of the (Na\(^+\),K\(^+\)) pump, stimulation of glucose uptake occurs primarily through a translocation of insulin-sensitive glucose transporters (Birnbaum, 1989) from an intracellular pool to the plasma membrane (Cushman and Wardzala, 1980; Karnieli et al., 1981; Suzuki and Kono, 1980), although other mechanisms could also contribute to the increase in uptake activity (Joost et al., 1988; Matthaei et al., 1988, Whitea and Adumrud, 1986). In contrast to the activity of the \(a2\) isoform of the (Na\(^+\),K\(^+\)) ATPase, the intrinsic glucose transport activity of adipocyte plasma membranes does not appear to be increased as compared to that of intact cells. However, in plasma membranes isolated from insulin-treated adipocytes, glucose transport activity is increased in comparison to that in plasma membranes isolated from untreated adipocytes (Avruch et al., 1972; Ludvigsen and Jarett, 1980; Pillion et al., 1978). In plasma membrane vesicles prepared from adipocytes treated with or without insulin, glucose transport cannot be increased by subsequent insulin treatment (Avruch et al., 1972; Pillion et al., 1978).

In contrast to adipocyte plasma membranes, adipocyte ghosts prepared by hypotonic lysis (Rodbell, 1967) have been reported to maintain some of the insulin sensitivity seen in whole cells (for example, insulin-sensitive glucose uptake) (Iliana and Cuatrecasas, 1971; Rodbell, 1967b). It has been previously shown by Clausen et al. (1969) that these ghosts...
are sealed well enough to exhibit ouabain inhibitable K+ uptake.

The question addressed here is whether insulin is capable of activating the (Na+,K+) pump in adipocyte ghosts. In the present study, it is demonstrated that these ghosts maintain both forms of the pump in an active, albeit an insulin-insensitive state. In addition, evidence is presented that the $K_{0.5Na+}$ of the $\alpha_1$ and $\alpha_2$ isoforms are identical under certain conditions. The ghosts do not behave as if insulin stimulated because glucose transport in ghosts prepared from insulin-treated adipocytes is increased in relation to transport measured in ghosts prepared from untreated cells. These ghosts, by several criteria, appear identical to plasma membranes except that they form sealed compartments. Because ghost $^{86}Rb^+/K^+$ uptake activity decreases over time after addition of KCl to ghosts preincubated without KCl and because the ratios of activities of the two isoforms of the (Na+,K+) pump change over time of pumping, the hypothesis is put forward that the apparent contribution of each isoform to the total pumping activity is affected by fast changes in the [Na+]. Since the uptake measurements. Because of the wide implications to our understanding of the mechanism of insulin activation of the adipocyte (Na+,K+) pump, this hypothesis was directly tested in adipocytes, and the results are presented in the accompanying article (McGill and Guidotti, 1991).

**EXPERIMENTAL PROCEDURES**

**Materials**—Male CD rats (125-175 g) were obtained from Charles River Breeding Laboratories. Collagenase (type I) from Cooper Biomedical and BSA (fraction V) from Armour Pharmaceutical Co. were both screened as previously described (Lyttot et al., 1985). Isotopes ($^{38}$RbCl, 2-deoxy[3H]glucose, [3H]cytochalasin B, [14C]sucrose) were obtained from Du Pont-New England Nuclear. Ion exchange resin, AG-50WXS, was from Bio-Rad. Ouabain was from Sigma. Porcine Zn++ Insulin was from Lilly. All other reagents were of analytical grade or better.

**Adipocyte, Ghost, and Membrane Preparation**—Adipocytes were prepared essentially according to Resh et al. (1980) except that the buffer was a Krebs-Ringer-HEPES (1-4% (w/v) BSA, 140 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgSO4, 10 mM HEPES) (KRH) buffer adjusted to pH 7.4 with Tris base.

Adipocyte ghosts were prepared essentially according to Rodbell (1967a) with minor modifications. Briefly, adipocytes prepared as above were rinsed quickly two times by gentle resuspension and centrifuging in a hypotonic lysis buffer consisting of 2.5 mM Na-ATP, 2.5 mM MgCl2, 0.1 mM CaCl2, 0.1 mM NAD, 1.0 mM HEPES adjusted to pH 7.4 with Tris base. An equal volume of hypotonic buffer at room temperature was added to the cells, and the mixture was rocked on a specimen mixer at 23 °C for 20 min. The suspension was centrifuged in a clinical centrifuge at 900 x g for 15 s. The supernatant and pellet were withdrawn, the pellet resuspended in 2 ml of hypotonic buffer, 2 ml of 600 mM sucrose/hypotonic buffer was added, and the suspension centrifuged again for 15 min as above. This pellet is the final ghost pellet and was resuspended in incubation buffer and placed on ice until used for the experiment. The ghosts were always prepared fresh for each experiment but can be kept on ice for several hours (as needed) without any noticeable change in the activity of the (Na+,K+) pump.

Adipocyte membranes were prepared as previously described (Resh, 1982). To prepare ghost plasma membranes, the final ghost pellet was resuspended in 4 ml of adipocyte homogenization buffer (Resh, 1982), sonicated for 2-5 s intervals, then homogenized 10 strokes with a motorized homogenizer at 12,000 rpm. The rest of the preparation was as for adipocytes.

$^{38}$RbCl/K+ Uptake Experiments—When ghosts were to be used for fixed [Na+] experiments (such as ouabain titration curves), the final ghost pellet was resuspended in KRH, 1% BSA, 0 mM KCl, with the appropriate [Na+], choline Cl to balance the salt to 140 mM. The ghosts were aliquotted to tubes with varying ouabain concentrations and kept on ice. Time equal to zero was considered the time at which the tube was placed at 37 °C. The ghosts were incubated with ouabain at 37 °C for 30 min at which time $^{42}$RbCl (4 μCi/ml) and KCl to 5 mM were added. Uptake was allowed to continue for the indicated amount of time. Uptake was terminated by passing an aliquot of ghosts over 0.7 ml of AG-50X3 ion-exchange resin that had been rinsed with three to four column volumes of 300 mM glycero1, 20 mM HEPES to pH 7.4 with Tris base). The ghosts were forced just into the resin with gentle air pressure, and rinsed through the column with two 0.5-ml aliquots of column buffer, also pushed through the column with air pressure. The amount of $^{86}$Rb not adsorbed to the column was determined in the eluates by the column method (McGill, 1990). A second aliquot of ghosts. The results obtained with the columns are comparable to results obtained by the pelleting method described by Rodbell (1967a) (data not shown), but the columns allowed quicker sample processing and dramatically increased the signal to noise ratio.

For experiments in which the [Na+] was varied, the final ghost pellet was resuspended (0.2-0.8 mg of protein/ml) in 1% BSA, KRH buffer with 0 mM each of NaCl, choline Cl, and KCl. Aliquots were quickly added to equal volumes of cold KRH with 2 X NaCl/choline Cl, 0 mM KCl. [NaCl + choline Cl] was kept constant at 150 mM. For determining the activity contributed by $\alpha_1$ and $\alpha_2$ at each [Na+], the ghosts were incubated with 0 mM, 3-10 μM, or 1 mM ouabain and specific activities calculated by subtraction as for adipocytes (Lytton, 1985). These samples were kept on ice until incubation was initiated by placing the tubes at 37 °C. Incubation and uptake were conducted as described above. When insulin was used in an experiment, it was always added to 10 nM 10 min prior to the addition of ouabain.

1-Deoxyglucose Uptake—Adipocytes were incubated ±90 nM insulin for 20 min prior to glucose transport. The final ghost pellet was resuspended in cold KRH at 0.1-0.4 mg of protein/ml. They were incubated for 30 min at 37 °C ±0.1 μM cytochalasin B. 1-Deoxy [3H]glucose (4-5 μCi/ml, 0.1 mM) was added and uptake measured for various time intervals. Uptake was terminated by placing 0.1 ml aliquots in 1 ml of cold KRH + 10 μM cytochalasin B and centrifuging at 12,000 X g for 30 s. The supernatant was aspirated off, and 1 ml of the cold KRH/cytochalasin B buffer was gently layered over the pellet. The sample was centrifuged for 15 additional s and the supernatant aspirated. The bottom portion of the Eppendorf tube containing the pellet was cut from the remainder of the tube. It was placed in 0.4 ml 5% sodium dodecyl sulfate for 30 min, and counted with Aquasol.

For experiments in which the effect of insulin on glucose transport in isolated ghosts was determined, insulin (10 nM) was added 10 min prior to the addition of 1-Deoxy [3H]glucose. [3H]Cytochalasin B Binding—The binding assay was essentially according to Wang (1985) with several modifications. The buffer used was a HEPES/Tris buffer instead of NaPO4 and [14C]sucrose was used to determine trapped volume in the pellets (Cushman and Wardzala, 1980). Briefly, ghost membranes (20-80 μg of membrane protein) were incubated with 0.1 μM cytochalasin B and aliquots were placed in 0.1 ml of either [3H]cytochalasin B (18.5 Ci/mmol, 20-120 μM) with [14C]sucrose (0.1 Ci/mmol, added to approximate counts of [H]) were added to the membranes and binding allowed to continue for 20 min at 25 °C. The membranes were centrifuged for 30 min at 12,000 X g (plasma membranes) or 90 min at 200,000 X g (microsomal membranes). Aliquots of the supernatant were counted and the rest aspirated. The pellets were processed as above except that they were counted in a Beckman scintillation counter using a dual-label dpm program to calculate actual [H] and [3H] dpm. [3H] dpm in the pellet were corrected for trapped volume estimated with [3H] dpm in the pellet.

**Statistical Analysis**—Nonlinear least squares analyses were performed on an AT&T AT computer using the RS/1 package from BBN Research Systems (Cambridge, MA). Curves for determining the Na+ dependence of (Na+,K+) pump activity were fit to the equation $V_{max} = (V \times [Na]^n)/(K_{Na} + [Na]^n)$ where $K_{Na} = K_{max} + [Na]$ (Lyttot, 1985).

Assays—Ghosts were centrifuged in column buffer and the pellet resuspended in water for later determination of protein. Protein was measured according to Peterson's modified Lowry technique using BSA as the standard (Peterson, 1977). 5'-Nucleotidase was measured according to Aronson and Touster (1974) with several modifications. The buffer used was 0.1 M Tris, 0.1 M KC1, 10 mM MgCl2, 1 mM dithiothreitol, 15 mM glycerol, 15 mM EGTA, 20 mM HEPES to pH 7.4 with Tris base). 5'-Nucleotidase was measured according to Aronson and Touster (1974) with several modifications. The buffer used was 0.1 M Tris, 0.1 M KC1, 10 mM MgCl2, 1 mM dithiothreitol, 15 mM glycerol, 15 mM EGTA, 20 mM HEPES to pH 7.4 with Tris base).
RESULTS

Ghost (Na+, K+) Pump—In order to determine if both forms of the (Na+, K+) pump are active in adipocyte ghosts, the ouabain dependence of $^{86}$Rb+/K+ pumping under conditions of maximal activity was measured. The ghosts were incubated with 140 mM NaCl, 0 mM KCl for 30 min at 37° C. Under these conditions in adipocytes, the (Na+, K+) pump is inactive and sodium equilibrates across the membrane (Lytton, 1985). Pumping was initiated by the addition of 140 mM NaCl with KC1 to a final concentration of 5 mM and uptake was quenched after 5 min. Fig. 1 shows the average of two such experiments. The (Na+, K+) pump in ghosts is very similar to that in adipocytes measured under identical conditions (data not shown) to that of insulin-treated adipocytes (Lytton, 1985), in that there are two ouabain inhibitable components: the α2 form, with a $K_i$ for ouabain of 1.3 x 10^-5 M and the α1 form with a $K_i$ for ouabain of 4.5 x 10^-6 M. The α2 isozyme contributes 63% of the total pumping activity, similar to the α2 contribution to total pumping activity in adipocytes under maximal pumping conditions (data not shown).

To determine insulin's effect on adipocyte ghosts, ouabain-inhibitable $^{86}$Rb+ uptake and [3H]-2-deoxyglucose uptake were determined with and without insulin as a measurement of activity of the (Na+, K+) pump and glucose transporter, respectively. The data in Table I demonstrate that insulin has no effect on the activity of either transport system in ghosts. In this respect, adipocyte ghosts are indistinguishable from adipocyte plasma membranes or cells already stimulated by insulin.

To determine whether the activities of the (Na+, K+) pumps in ghosts resemble those in isolated plasma membranes, adipocytes, or insulin-stimulated adipocytes, the activity of the (Na+, K+) pump in ghosts and adipocytes was measured at a lower internal [Na+] to achieve submaximal activity. Ghosts and adipocytes were incubated with 50 mM NaCl, 100 mM choline Cl (to balance osmotic strength), 0 mM KCl for 30 min; $^{86}$Rb+/K+ uptake was measured for 5 min as described under "Experimental Procedures." Data are expressed as % maximum with 3 mM (ghosts) or 5 mM (adipocytes) ouabain residual activity subtracted. Ghosts: data are averages ± S.E. of two experiments. $K_i$ values for ouabain are 3.0 ± 0.3 x 10^-5 M for α2 and 0.89 ± 0.11 x 10^-5 M for α1. The α2 isozyme accounts for 62 ± 1% of total activity. $r^2$ = 0.999. Adipocytes: data are averages ± S.E. of three experiments. $K_i$ values for ouabain are 2 ± 5 x 10^-7 M for α2 and 1.4 ± 0.6 x 10^-7 M for α1. The α2 isozyme accounts for 16 ± 8% of total activity. $r^2$ = 0.972.

Fig. 2. Ouabain dependence of $^{86}$Rb+/K+ pumping in adipocytes and adipocyte ghosts under conditions of submaximal activity. Ghosts and adipocytes were incubated with 50 mM NaCl, 190 mM choline Cl, 0 mM KCl for 30 min and $^{86}$Rb+/K+ uptake measured for 5 min as described under "Experimental Procedures." Data are expressed as % maximum with 3 mM (ghosts) or 5 mM (adipocytes) ouabain residual activity subtracted. Ghosts: data are averages ± S.E. of two experiments. $K_i$ values for ouabain are 3.0 ± 0.3 x 10^-5 M for α2 and 0.89 ± 0.11 x 10^-5 M for α1. The α2 isozyme accounts for 62 ± 1% of total activity. $r^2$ = 0.999. Adipocytes: data are averages ± S.E. of three experiments. $K_i$ values for ouabain are 2 ± 5 x 10^-7 M for α2 and 1.4 ± 0.6 x 10^-7 M for α1. The α2 isozyme accounts for 16 ± 8% of total activity. $r^2$ = 0.972.

It was next necessary to determine the sodium dependence of $^{86}$Rb+/K+ uptake into ghosts in a manner similar to that used for adipocytes (Lytton, 1985) to determine the apparent Na+ affinities of both isozymes of the pump. The $K_{on}$Na of...
each isofrom of the pump gives an indication of the state of the enzyme in that in isolated plasma membranes the two isoforms have indistinguishable $K_{\text{0.5Na}^+}$, whereas in insulin-stimulated adipocytes, the $K_{\text{0.5Na}^+}$ for the $a_2$ isozyme is two to three times greater than for the $a_1$ isozyme (Lytton, 1985). The activity of each isozyme can be measured independently in the same system because each isozyme has a different $K_v$ value for the inhibitor ouabain. These $K_v$ values are sufficiently different such that at intermediate concentrations of ouabain (3 μM) only the $a_2$ isozyme is inhibited (see Fig. 1).

To use a subtraction method to estimate the activities of each isozyme at various $Na^+$ concentrations, the $K_v$ values for ouabain of each isofrom must remain constant under all conditions used. The data in Table II illustrate that the $K_v$ values for ouabain for both isoforms do indeed remain constant under all conditions tested, validating the use of the subtraction method in estimating the activities of two $Na^+/K^+$ pump isoforms in the same system.

The $Na^+$ dependence of $^{86}Rb^+$ pumping activities of the two isoforms is shown in Fig. 3. The $K_{\text{0.5Na}^+}$ values obtained from the curve fits are 14.5 mM and 29.0 mM for the $a_1$ and $a_2$ isoforms, respectively. The $K_{\text{0.5Na}^+}$ value for the $a_1$ isozyme in ghosts is in good agreement to those described in adipocytes, insulin-treated adipocytes, and adipocyte plasma membranes, consistent with its role as the "constitutive" form of the pump (Lytton, 1985). However, the $K_{\text{0.5Na}^+}$ value obtained for the $a_2$ isozyme is most similar to that observed in insulin-treated adipocytes (Lytton, 1985 and data not shown) and not to that of isolated plasma membranes. Neither treatment of cells with insulin before ghost preparation nor treatment after preparation had any effect on the $Na^+$ affinity of either form of the pump in ghosts (data not shown).

Glucose Transporter—These results suggest that the $a_2$ isofrom of the ($Na^+,K^+$) pump in ghosts is in a state similar to the isoform in insulin-stimulated state and could perhaps explain the lack of insulin effect demonstrated in Table I. The question, then, is whether the putative stimulation is specific for the ($Na^+,K^+$) pump, or if it also involves the glucose transport system. If ghost preparation causes a translocation of glucose transporters to the plasma membrane, one would expect treatment of adipocytes with insulin prior to ghost preparation to have no effect on the location of glucose transporters. Conversely, if treatment with insulin prior to ghost preparation does cause a change in cellular location of transporters, one can assume that the preparation technique does not stimulate glucose transport in a way that mimics insulin action. D-

**Table II**

**Determination of the $K_v$ values for ouabain for the $a_1$ and $a_2$ isoforms of the ghost ($Na^+/K^+$) pump under different conditions**

Ghosts were incubated with various ouabain concentrations in KClH with different sodium concentrations, choline chloride to balance osmolarity, with or without 5 mM KCl for 30-60 min. $^{86}Rb^+$ was added alone or with KCl (5 mM) and uptake measured at 5 min. The $K_v$ values for ouabain for each isofrom were determined as for Fig. 1. Shown are the values from single and multiple determinations ± range ($n = 2$) or S.E. ($n > 2$).

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>$K_v$ values for ouabain $n$</th>
<th>$a_2$</th>
<th>$a_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NaCl] [KCl]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mM</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 0 2</td>
<td>3.1 ± 1.0 10^-7</td>
<td>2.0 ± 0.0 10^-4</td>
<td></td>
</tr>
<tr>
<td>50 0 2</td>
<td>2.5 ± 0.5 10^-7</td>
<td>1.2 ± 0.3 10^-4</td>
<td></td>
</tr>
<tr>
<td>75 0 1</td>
<td>2.5 ± 10^-7</td>
<td>2.5 ± 10^-4</td>
<td></td>
</tr>
<tr>
<td>125 0 4</td>
<td>3.1 ± 1.7 10^-7</td>
<td>1.0 ± 0.4 10^-4</td>
<td></td>
</tr>
<tr>
<td>20 5 1</td>
<td>5.0 ± 10^-7</td>
<td>2.8 ± 10^-4</td>
<td></td>
</tr>
<tr>
<td>50 5 1</td>
<td>7.9 ± 10^-7</td>
<td>3.5 ± 10^-4</td>
<td></td>
</tr>
<tr>
<td>125 5 2</td>
<td>5.8 ± 4.0 10^-7</td>
<td>0.8 ± 0.0 10^-4</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.** $[Na^+]$ dependence of $^{86}Rb^+/K^+$ uptake into ghosts. Ghosts (0.1-0.4 mg/ml) were incubated with 0 mM, 3 μM, or 1 mM ouabain at various $[Na^+]$, 0 mM KCl, and $^{86}Rb^+/K^+$ uptake measured for 5 min as described under "Experimental Procedures." $a_1$ and $a_2$ activities were calculated by subtraction as described (Lytton, 1985). The data shown are the averages ± S.E. of three experiments. The maximal values used to calculate % maximum activity are the $V_{\text{max}}$ values from the curve fits of $a_1$ and $a_2$ activities for each experiment. $a_1$, $K_{\text{0.5Na}^+} = 14.5 ± 1.1$ mM, $f^2 = 0.991$. $a_2$, $K_{\text{0.5Na}^+} = 29.0 ± 2.6$ mM, $f^2 = 0.980$.

**Table III**

**Measurement of $D$-glucose inhibitable $[^3H]cytochalasin B binding in membranes**

Specific $D$-glucose inhibitable binding to membranes was determined as described under "Experimental Procedures." Shown is the individual data of three ghost experiments and one adipocyte experiment where each point was determined in duplicate. Insulin treatment was always conducted on adipocytes either before isolation of membranes or before preparation of ghosts from which membranes were then isolated.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pm $[^3H]cytochalasin B$ bound/mg membrane protein</th>
<th>[Cytochalasin B]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghost 1</td>
<td>1.0 ± 0.1 0.2 ± 0.1 ND (ND)</td>
<td>28</td>
</tr>
<tr>
<td>Ghost 2</td>
<td>3.4 ± 1.0 1.9 ± 0.5 0.1 ± 3.9 6.3 ± 2.7</td>
<td>71</td>
</tr>
<tr>
<td>Ghost 3</td>
<td>1.1 ± 0.7 0.3 ± 0.2 ND ND</td>
<td>37</td>
</tr>
<tr>
<td>Adipocyte</td>
<td>3.7 ± 0.5 0.1 ± 0.9 2.6 ± 0.8 4.1 ± 0.5</td>
<td>66</td>
</tr>
</tbody>
</table>

5'-Nucleotidase activity

<table>
<thead>
<tr>
<th>$\mu M/mg/h$</th>
<th>2.6</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase activity</td>
<td>1.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

$^a$ Plasma membranes.

$^b$+/− insulin treatment of adipocytes.

$^c$ Microsomal membranes.

$^d$ Not determined.

Glucose-inhibitable $[^3H]cytochalasin B$ binding to ghost membranes prepared from cells that initially were treated with or without insulin for 20 min was used to determine membrane location of glucose transporters. As shown in Table III, in three experiments, little or no $D$-glucose-inhibitable $[^3H]cytochalasin B$ binding was demonstrable in ghost plasma membranes without insulin treatment of cells, but increased amounts of binding were measurable in plasma membranes if the cells were treated with insulin prior to preparation of ghosts. The unchanged distribution of 5'-nucleotidase activity (normally enriched in plasma membranes) indicates that, as observed by others (Cushman and Wardzala, 1980), the overall fractionation pattern of the membranes is unaffected by insulin treatment of cells. Because much of the microsomal
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material is lost upon lysis (data not shown), it is difficult to obtain enough material to measure cytochalasin binding accurately to these membranes. In those experiments where some material was obtained, a decrease in binding was observed when the cells were first treated with insulin, but the errors were large, presumably due to the very small amount of material available.

Consistent with the [3H]cytochalasin binding data are 2-deoxyglucose uptake experiments in which uptake is measured in ghosts prepared from adipocytes treated with or without insulin (Fig. 4). Uptake activity in ghosts prepared from cells pretreated with insulin is 2.4-fold higher than in control ghosts. The data shown are the averages of material available. In those experiments where pretreated with insulin is 2.4-fold higher than in control ghosts. The data shown are the averages of material available.

Intra-ghost [Na⁺]—The results presented above suggested that the preparation of adipocyte ghosts results in partial activation of the two isozymes of the (Na⁺,K⁺) pump and has no effect on the glucose transport system. This result with two insulin-responsive membrane transporters which ordinarily respond similarly to insulin and insulin-like stimuli, was unexpected; it encouraged a re-evaluation of the "Rb+/K⁺ uptake at all times less than 5 min would be identical to that described in adipocyte plasma membranes, where the α2 isozyme has a high apparent sodium affinity, rather than like that described in insulin-treated adipocytes, where the α2 isozyme has an intermediate value for Kₐₐₛₚ. The conclusion is that the difference in the Na⁺ dependences of the two forms of the ghost (Na⁺,K⁺) pump shown in Fig. 3 is an artifact caused by a rapid decrease in intravesicular Na⁺ concentrations during the 5 min of incubation in the presence of KCl.

In order to obtain an estimate of the steady state [Na⁺], reached when the adipocyte ghosts containing high [Na⁺], are allowed to pump normally, the ouabain dependence of "Rb⁺/K⁺ uptake was determined as a function of time after transfer to a media containing 5 mM KCl. Shown in Fig. 6 is one representative experiment where ghosts were incubated in KRH with 150 mM NaCl, 0 mM KCl, and various ouabain concentrations. KCl (5 mM) was added and "Rb⁺/K⁺ uptake was measured for 5 min at T = 0, 15, or 30 min after the addition of KCl as described under "Experimental Procedures." The total (Na⁺,K⁺) pump activity (measured at 0 mM ouabain) has decreased by 70% after 30 min, suggesting that the [Na⁺], has decreased to less than 10 mM.

One should note that the Kₐ values for ouabain remain

![Diagram](image-url)

**Fig. 4.** Effects of insulin treatment of adipocytes on 2-deoxyglucose uptake in ghosts. Cytochalasin B-inhibitable 2-deoxyglucose uptake was measured as described under "Experimental Procedures." O, ghosts prepared from insulin-treated adipocytes. △, control ghosts. The data shown are the averages ± S.E. of two experiments expressed as % maximum. The maximum value of each experiment was taken as the 12-min time point, + insulin.

![Diagram](image-url)

**Fig. 5.** The dependence of apparent Kₐₛₚ on the time of uptake after addition of KCl to ghosts. The ghosts were incubated and uptake measured as in Fig. 3 except that after addition of "Rb⁺/K⁺", aliquots were taken at 1.5, 3.5, and 5 min and uptake terminated as described. Shown is the average ± S.E. of two experiments. The maximal values used for calculating % maximal activity were those V₉₅ values obtained from the curve fits. ○ = 1.5-min uptake; △ = 3.5-min uptake; Δ = 5-min uptake. Kₐₛₚ given in mM Na⁺. V₉₅ given in mM Na⁺ taken up/min/mg ghost protein. Panel A, α2 isozyme activity: 1.5': Kₐₛₚ = 11.4 ± 1.6, V₉₅ = 4.0 ± 0.2, r² = 0.989. 3.5': Kₐₛₚ = 18.4 ± 2.3, V₉₅ = 4.0 ± 0.2, r² = 0.987. 5': Kₐₛₚ = 28.0 ± 2.6, V₉₅ = 3.2 ± 0.2, r² = 0.979. Panel B, α1 isozyme activity: 1.5': Kₐₛₚ = 2.2 ± 3.8, V₉₅ = 1.7 ± 0.2, r² = 0.833. 3.5': Kₐₛₚ = 8.6 ± 1.3, V₉₅ = 2.0 ± 0.1, r² = 0.977. 5': Kₐₛₚ = 14.1 ± 1.0, V₉₅ = 1.5 ± 0.1, r² = 0.985.
constant over time, again proving the validity of using a subtraction method in determining the activity of each isozyme independently, as done in Figs. 3 and 5. Surprisingly, however, with increasing time of pumping (thus, decreasing internal \([\text{Na}^+]\)) the apparent activities contributed by the \(a1\) and \(a2\) isozymes do not decrease equally, but rather the activity of the \(a2\) form of the pump seems to be much more sensitive to the increasing times of incubation with KCl. The likely explanation is that with increasing times of incubation with KCl at low ouabain concentrations, where very little of the pump is inhibited, ghosts would experience a very dramatic decrease in \([\text{Na}^+]\), and thus a smaller decrease in pumping activity. In contrast, ghosts incubated at higher ouabain concentrations, where much of the pump is inhibited, would experience very little change in \([\text{Na}^+]\) over time, and thus a smaller decrease in pumping activity. This hypothesis could explain the disparity seen in the decrease of activity of both forms of the \((\text{Na}^+,\text{K}^+)\) pump. However, it would require measuring the \([\text{Na}^+]\), at all ouabain concentrations to directly prove or disprove the idea that \([\text{Na}^+]\) changes during the experiment.

DISCUSSION

The mechanism by which insulin stimulates the activity of the \((\text{Na}^+,\text{K}^+)\) pump in rat skeletal muscle and adipocytes is not known. On the basis of the differential affinity of the isoforms of the \((\text{Na}^+,\text{K}^+)\) ATPase for ouabain, Resh et al. (1980) and Lytton et al. (1985) have suggested that only the \(a2\) isoform is affected by insulin. In addition, Lytton (1985) has presented evidence that in rat adipocytes the fractional activity of the \(a2\) isoform is lower than that of the \(a1\) isoform because the apparent \(K_{0.5,\text{Na}^+}\) of the \(a2\) isoform is larger than that of the \(a1\) isoform. The \(K_{0.5,\text{Na}^+}\) of the \(a2\) isoform is similar to that of the \(a1\) isoform in isolated adipocyte plasma membranes, and approaches that of the \(a1\) isoform in insulin-treated adipocytes.

The key to understanding the problem is to uncover the mechanism that brings about the selective decrease in the activity of the \(a2\) isoform of the \((\text{Na}^+,\text{K}^+)\) pump in unstimulated adipocytes.

Since attempts to affect the activity of the \(a2\) isoform of the \((\text{Na}^+,\text{K}^+)\) ATPase by addition of cytoplasmic fractions of adipocytes to plasma membranes were not successful (data not shown), it was considered that in adipocyte ghosts, produced by gentle lysis of the cells (Rodbell, 1967a), the \((\text{Na}^+,\text{K}^+)\) pump might retain the properties that it has in cells and allow investigation of the phenomenon. In fact, in adipocyte ghosts both isoforms of the \((\text{Na}^+,\text{K}^+)\) pump are active and pump \(K^+\) into the cytoplasmic space in an ouabain-inhibitable manner in the presence of a high intravesicular \([\text{Na}^+]\), as is shown in Fig. 1. In ghosts, however, insulin does not have an effect on the activity of the \((\text{Na}^+,\text{K}^+)\) pump or the glucose transport system. There are reports that insulin does stimulate glucose uptake in adipocyte ghosts; the effects, however, are small and sporadic (Iliano and Cuatrecasas, 1971; Rodbell, 1967b).

Comparison of the activities of the isoforms of the \((\text{Na}^+,\text{K}^+)\) pump in adipocytes and ghosts under similar conditions revealed distinct differences in the properties of the \(a2\) isoform. The results shown in Figs. 2 and 3 indicate that the activity of the \(a2\) isoform in ghosts is greater than that in cells at the same initial intracellular \([\text{Na}^+]\) and that the apparent \(K_{0.5,\text{Na}^+}\) of this isoform in ghosts is smaller than that in cells. Thus, the preparation of ghosts appears to have an effect similar to that of insulin on the properties of the \(a2\) isoform of the \((\text{Na}^+,\text{K}^+)\) pump, a conclusion supported by the lack of effect of insulin on the activity of the \((\text{Na}^+,\text{K}^+)\) pump in these ghosts. This result can be explained in two ways. One possibility is that the hypotonic lysis used in the preparation of ghosts actually activates the intracellular signaling system coupled to the insulin receptor, as appears to be the case with hyperosmolarity (Claussen et al., 1970). The other hypothesis is that the inhibitory machinery present in cells which inactivates the pump has been completely lost during the preparation of ghosts or plasma membranes.

To distinguish between these two possibilities, the effect of ghost preparation on the activity of the glucose transport system was studied (Karnieli et al., 1981). If the preparation of ghosts activates the insulin signaling system, translocation of insulin-regulated glucose transporters from microsomal membranes to the plasma membrane would take place; thus, the number of glucose transporters in the plasma membrane of ghosts should be greater than that in the plasma membrane of cells, and similar to that in the plasma membrane of insulin-treated cells. The results shown in Fig. 4 and Table III show that ghost preparation does not have insulin-like effects on the glucose transport system. The conclusion is that the preparation of ghosts results in a loss in cytoplasmic components, including microsomal vesicles containing insulin-regulated glucose transporters and the regulatory machinery for the \((\text{Na}^+,\text{K}^+)\) pump, so that the ghosts are identical to sealed plasma membranes.

If this conclusion is correct, it seems odd that the properties of the \(a2\) isoform of the \((\text{Na}^+,\text{K}^+)\) pump in ghosts are different from those of this isoform in membranes; that is, it was unexpected that the \(K_{0.5,\text{Na}^+}\) of the \(a2\) isoform should have different values in the two situations. This peculiarity led to the idea that the difference in the \(K_{0.5,\text{Na}^+}\) values might be apparent and a consequence of the fact that the ghosts contain a sealed space while membranes do not. With membranes, the concentrations of ions is constant during the assay of activity; on the other hand, the internal space of sealed ghosts
is small and the concentrations of Na$^+$ and K$^+$ are likely to change during the time used for the uptake. In fact, when the Na$^+$ dependence of $^{86}\text{Rb}^+$/K$^+$ uptake was determined as a function of the time used for the measurement, it was found that the values for the $K_{o,Na}$ of both isoforms decreased as the time of the measurement decreased; the values for the a1 and a2 isoforms were identical at the shortest time for uptake. These results confirm the view that in adipocyte ghosts, the properties of both isoforms of the (Na$^+$,K$^+$) pump are similar to those in membranes; the apparent $K_{o,Na}$ of the a2 isoform is different from that in adipocytes. The simplest explanation for the results with ghosts is that the intravesicular [Na$^+$] values are measurable in adipocytes, allowing pumping activity, even at low [Na$^+$]; the intravesicular [Na$^+$] changes as a function of the ouabain concentration.

The apparent $K_{o,Na}$ in the a1 isoform is insensitive to insulin and because its activity is increased with the increasing time of exposure of the ghosts to K$^+$, a condition which brings about a decrease in the internal [Na$^+$]. Thus, either the dependence of the activity of the a2 isoform differs from that of the a1 isoform, or the ouabain dependence curve does not give the correct estimate of the relative activities of the isoforms. The latter situation might result if the intravesicular [Na$^+$] changes as a function of the ouabain concentration, giving an illusory high value for the activity of the a1 or low ouabain affinity form of the pump. If this is true, ouabain dependence curves of pump activity could not be used to estimate the ratios of activity contributed by different isoforms of the (Na$^+$,K$^+$) pump unless the true Na$^+$ dependencies and internal Na$^+$ concentrations are also known. Unfortunately, it is not possible in ghosts to measure [Na$^+$], accurately.

The present work shows that the (Na$^+$,K$^+$) pump in adipocyte ghosts behaves identically to the (Na$^+$,K$^+$) pump described in adipocyte plasma membranes based on three criteria: 1) the a2 isoform contributes greater than 50% of total pumping activity, even at low [Na$^+$]; 2) both isoforms have similar $K_{o,Na}$ values when activities are measured quickly after reactivation of the pump; and 3) both forms of the (Na$^+$,K$^+$) pump are insensitive to insulin activation. The ghost glucose transporter also appears to behave identically to the plasma membrane glucose transporter both because it is insensitive to insulin and because its activity is increased in fractions prepared from insulin-treated cells as compared to those prepared from untreated cells.

The ghost system, although not the insulin-sensitive subcellular system for which one might have hoped, has proved to be a useful, manipulatable tool providing new insights on the insulin regulation of the (Na$^+$,K$^+$) pump. Some of the questions raised by this work on the ghost system need to be addressed using intact adipocytes for two reasons. First, internal [Na$^+$] values are measurable in adipocytes, allowing questions concerning these values to be addressed directly rather than indirectly. Second, and most importantly, the original interpretations of adipocyte experiments were made with the assumptions that [Na$^+$], is constant both during uptake after addition of KCl to K$^+$ starved cells and at all ouabain concentrations. If these assumptions are not valid in adipocytes, the isozyme specificity of insulin stimulation of the (Na$^+$,K$^+$) pump needs to be reappraised.

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