Insulin Stimulation of (Na⁺,K⁺)-Adenosine Triphosphatase-dependent ⁸⁶Rb⁺ Uptake in Rat Adipocytes*

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Insulin stimulated the uptake of ⁸⁶Rb⁺ (a K⁺ analog) in rat adipocytes and increased the steady state concentrations of intracellular potassium. Half-maximal stimulation occurred at an insulin concentration of 200 pm. Both basal- and insulin-stimulated ⁸⁶Rb⁺ transport rates depended on the concentration of external K⁺, external Na⁺, and were 96% inhibited by 10⁻³ M ouabain and 10⁻³ M KCN, indicating that the hormone was activating the (Na⁺,K⁺)-ATPase. Insulin had no effect on the entry of ²²Na⁺ or exit of ⁸⁶Rb⁺. Kinetic analysis demonstrated that insulin acted by increasing the maximum velocity, Vₘₐₓ, of ⁸⁶Rb⁺ entry. Inhibition of the rate of Rb⁺ uptake by ouabain was best described by a biphasic inhibition curve. Scatchard analysis of ouabain binding to intact cell indicated binding sites with multiple affinities. Only the rubidium transport sites which exhibited a high affinity for ouabain were stimulated by insulin. Stimulation required insulin binding to an intact cell surface receptor, as it was reversible by trypsinization. We conclude that the uptake of ⁸⁶Rb⁺ by the (Na⁺,K⁺)-ATPase is an insulin-sensitive membrane transport process in the fat cell.

The sodium and potassium ion-activated adenosine triphosphatase is the enzyme responsible for active transport of sodium and potassium ions and the maintenance of a cationic gradient across the plasma membrane of almost all eukaryotic cells (for review, see Ref. 1). The activity of this membrane-bound transport protein is specifically inhibited by the cardiac glycoside ouabain, which binds to the portion of the enzyme facing the extracellular fluid (2) and thereby inhibits ion transport and ATP hydrolysis.

There are numerous reports in the literature that the levels of intracellular Na⁺ and K⁺ are altered by insulin. As early as 1924, it was observed that the levels of serum potassium decreased in response to administration of insulin in vivo (3). More recent in vitro studies document insulin stimulation of K⁺ uptake (4–8) and Na⁺ efflux (8–12) in various preparations of intact frog and rat muscle, as well as rat adipose tissue (13), mouse fat cells (14), and duck salt gland (15). In several cases the insulin effect was shown to be inhibited by cardiac glycosides (7, 8, 10–12, 14, 15), suggesting that insulin affects the transport activity of the (Na⁺,K⁺)-ATPase. However, no single study has demonstrated that physiological concentrations of insulin stimulate potassium transport, that this effect is mediated by the (Na⁺,K⁺)-ATPase and correlates with insulin binding to its cell surface receptor.

The rat adipocyte is an isolated cell type in which the binding of insulin to its receptor and the effects of the hormone on the membrane transport system for glucose have been studied in great detail (16–18). We, therefore, decided to investigate the effects of insulin on ion transport in the fat cell in order to further define the kinetic parameters altered by hormonal stimulation of the (Na⁺,K⁺)-ATPase, to correlate the insulin effect with binding to the cell surface receptor, and to determine the Km of insulin on K⁺ transport in the intact isolated cell. In addition, we wondered whether there were similarities in the way insulin affects the transport systems for glucose and K⁺ in the same cell.

This paper demonstrates that insulin stimulated the uptake of rubidium (a potassium analog) into rat adipocytes and that this increased uptake was a consequence of the increased activity of the (Na⁺,K⁺)-ATPase. The stimulation of ⁸⁶Rb⁺ uptake serves as another criterion for insulin responsiveness in the intact cell.

EXPERIMENTAL PROCEDURES
Isolation of Fat Cells—Adipocytes were prepared according to the method of Rodbell (20). Epididymal fat pads were removed from 150- to 200-g male rats (CD strain, Charles River Breeding Laboratories) immediately following decapitation. Digestion was carried out in Krebs-Ringer phosphate buffer, containing 4% (w/v) bovine serum albumin and 1 mg/ml of bacterial collagenase (type I). The buffer contained 140 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1.0 mM MgSO₄, 10 mM NaHPO₄, and was adjusted to pH 7.4 with NaOH after addition of albumin. Following a 1-h digestion at 37°C in a shaking water bath, the cells were filtered through Japanese silk (8xx mesh) and washed three times with the albumin buffer in an International clinical centrifuge.

Transport Assays—The adipocyte suspension was made up to a 30 to 40% packed cell volume in round bottom polypropylene tubes. All transport assays were performed at 37°C in Krebs-Ringer phosphate albumin buffer, unless otherwise stated. Following the addition of tracer amounts of radioisotope (final concentration, 2 to 10 µCi/ml), duplicate 100-µl aliquots of cell suspension were removed at the indicated time intervals. Separation of cells from radioactive medium was achieved using the oil centrifugation technique of Gliemann et al. (21). The cell suspension was centrifuged through dextran polyethylene tube for 20 s in a Beckman Microfuge B. The tube was then frozen in a dry ice/acetone mixture and sliced through the oil layer. The cell layer and aqueous "subnatant" were placed in separate 20-ml scintillation vials and solubilized by vortexing in 0.4 ml of 5% sodium dodecyl sulfate. After 1 h, 4 ml of Aquasol (New England Nuclear) was added, and the vials were capped, vortexed, and counted in a Beckman liquid scintillation counter.

For determination of the Km of "Rb⁺ transport, fat cells were washed three times with K⁺-free buffer and resuspended in buffer containing the indicated concentration of RbCl. All tubes contained 0.5 ml of 30% cells and were preincubated for 10 min at 37°C in the presence or absence of 8 mM insulin. Transport was initiated by the addition of 5 µl of "RbCl. Ten minutes later, triplicate samples were counted.

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100-μl aliquots were withdrawn and processed as described above.

To determine the rate of $^{22}$Na efflux, 1.0 ml of packed fat cells (prepared in Krebs-Ringer phosphate buffer) was incubated with 20 μCi of $^{22}$NaCl for 10 min at 37°C. Insulin and/or ouabain was added during the final 2 min of the incubation. Following the addition of 2.0 mM NaCl-free buffer (140 mM choline chloride/5 mM KCl/1.4 mM CaCl$_2$/1 mM MgSO$_4$/10 mM Tris-phosphate/4% bovine serum albumin, pH 7.4) (choline Ringer), the cells were spun in an International clinical centrifuge and resuspended in 1.5 ml of choline Ringer with or without ouabain and/or insulin. The final cell suspension contained 2.5 ml of 40% adipocytes. Washing and resuspension of the cells was accomplished in 30 to 40 s. Time zero was defined as the time of addition of the 1.5 ml of choline Ringer. $^{22}$Na$^+$ transport was assayed at 37°C as described above. Alternatively, 0.5 ml of packed fat cells was loaded with $^{22}$Na as above, and 4 ml of choline Ringer were added at time zero. Both methods yielded nearly identical results.

Intracellular water volume and extracellular trapped volume measurements were made on suspensions of adipocytes containing 1 to 5 μCi/ml of $[^3]$H$_2$O and $[^14]$C)sucrose. These determinations were always made in parallel with experimental tubes containing $^{86}$Rb$^+$/[$^3$]H$_2$O or $^{22}$Na$^+$/[$^3$]H$_2$O. The spillover of $^{86}$Rb$^+$ or $^{22}$Na$^+$ into the set $^3$H-channel was less than 2%.

Ouabain-binding Analysis—Cells were placed in tubes containing 4 nM insulin or an equivalent volume of buffer and incubated at 37°C for 10 min. Then each tube received $1 \times 10^{-7}$ M [$^3$]H_ouabain and nonradioactive ouabain in a concentration range of $5 \times 10^{-7}$ M to $1 \times 10^{-6}$ M. Following a 35-min incubation at 37°C (a time sufficient to reach equilibrium), duplicate 500-μl aliquots were withdrawn from all tubes and processed as described.

Reagents—Collagenase (type I, 150 units/mg, batch 485014P) and trypsin-1-1-tosylamide-2-phenylethyl chloromethyl ketone were obtained from Worthington Biochemical Corp. Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Co. Dicyonyl phthalate was obtained from ICN-K&K Laboratories, Inc. Rubidium chloride was from Fisher Scientific Co. Ouabain and porcine insulin were from Sigma Chemical Co. Monensin was a gift from Stanley Goldin, Harvard Medical School. $^{86}$RbCl, $^{42}$KCl, $^{131}$I-insulin, $[^3]$H$_2$O, $[^14]$C)sucrose, and $[^3]$H$_2$O ouabain were from New England Nuclear. $[^1]$C)Arabinose was purchased from American. See Table I for the best fit of the data to a saturation kinetics curve by regression analysis (correlation coefficient $r = 0.98$). In A, the difference between filled and open circle raw data points is statistically significant ($p < 0.005$), whereas the difference between filled and open triangles was not ($p > 0.10$) (paired Student $t$ test).

**RESULTS**

**Insulin Effect on Rb$^+$ Transport**—Transport activity of the (Na$^+$,K$^+$)-ATPase can be monitored with radioactive rubidium ($^{86}$Rb$^+$), which substitutes for potassium in the activation of ATP hydrolytic activity and the active transport of K$^+$ into the cell (22). The time course of $^{86}$Rb$^+$ uptake by rat adipocytes is shown in Fig. 1. Addition of insulin (8 nM) caused a 50% increase in the rate of rubidium uptake (rate increase, 50% ± 20% in 33 experiments). This increase was apparent within 2 min after exposure to insulin, and it occurred when insulin was added to cells before, at the same time as, or 30 min after $^{86}$Rb$^+$ addition. The fractional increase in the rate of $^{86}$Rb$^+$ uptake was independent of the time of addition of insulin. Identical results were obtained when $^{42}$K$^+$ was used as the tracer. However, due to its longer isotopic half-life (19 days for $^{86}$Rb$^+$ versus 12 h for $^{42}$K$^+$), $^{86}$Rb$^+$ was far more convenient to handle and was used for all subsequent studies.

The uptake of $^{86}$Rb$^+$ shown in Fig. 1B exhibited saturation kinetics, indicating that the system was approaching isotopic steady state. We calculated the steady state level of K$^+$ in the cell based on the amount of $^{86}$Rb$^+$ which accumulated at saturation (Fig. 1B) and the intracellular water volume (determined as the net [$^3$]H$_2$O volume in the presence of [$^14$C)sucrose. In four separate experiments, the intracellular K$^+$ concentration was 177 mM (±27 mM, S.D.) in control untreated cells and 7 mM (±2 mM) in the presence of 1 mM ouabain; insulin increased this value to 222 mM (±26 mM). Thus the 50% stimulation of the initial rate of rubidium uptake induced by insulin resulted in an increase in the steady state K$^+$ concentration of roughly 40 mM.

If the insulin effect is mediated through the (Na$^+$,K$^+$)-ATPase, then stimulation should be abolished by ouabain, a specific inhibitor of the cation pump. The data in Fig. 1B represent the best fit of the data to a saturation kinetics curve by regression analysis (correlation coefficient $r = 0.98$). In A, the difference between filled and open circle raw data points is statistically significant ($p < 0.005$), whereas the difference between filled and open triangles was not ($p > 0.10$) (paired Student $t$ test).
An increase in enzyme activity can be mediated by a change in the Michaelis constant, \( K_m \), or maximum velocity, \( V_{max} \), or both. Fig. 2 demonstrates that the \( K_m \) of the \((Na^+K^+)-ATPase\) for \( Rb^+ \) (1.2 mM) remained unchanged in the presence of insulin but that the \( V_{max} \) of \( Rb^+ \) transport was increased. This effect was not a response to an insulin-induced increase in \( K^+ \) efflux from the cell. When cells were preincubated with \(^{86}Rb^+\), and ouabain was then added, the leakage of \( Rb^+ \) out of the cell was quite slow (Fig. 3). Insulin had no apparent effect on the exchange of \( Rb^+ \) for external \( K^+ \) during the time course monitored. Furthermore, if fat cells were preincubated with \(^{86}Rb^+\), then resuspended in \( K^-\)-free buffer, insulin had no observable effect on the efflux of \( Rb^+ \).

**Adipocyte \( Na^+ \) Fluxes**—To test the possibility that insulin increases the entry of \( Na^+ \) into the cell, the uptake of \(^{22}Na^+\) was monitored after the addition of insulin. Ouabain was present to prevent efflux of \( Na^+ \) through the \((Na^+,K^+)-ATPase\). No difference in the rate or amount of \(^{22}Na^+\) entering the adipocyte in the presence or absence of hormone was observed (Fig. 4A). Furthermore, when ouabain and insulin were added simultaneously (at time zero) to the adipocyte suspension, 

\[ \text{Table I} \]

| Effect of insulin and ATPase inhibitors on \(^{86}Rb^+\) uptake |
|---------------------------------------------------------------|
| A 30% adipocyte suspension was incubated for 15 min at 37°C in  |
| the absence or presence of the indicated compounds. Transport |
| was initiated by the addition of 2 \( \mu \)Ci of \(^{86}RbCl\). Ten |
| minutes later, triplicate 100-\( \mu \)l aliquots were withdrawn |
| and processed. Rate of \(^{86}Rb^+\) uptake | % |
| Control | 100 |
| 8 \( \mu \)M insulin | 150 |
| 1 \( \mu \)M ouabain | 10 |
| 1 \( \mu \)M ouabain + 8 \( \mu \)M insulin | 10 |
| 1 \( \mu \)M KCN | 12 |
| 1 \( \mu \)M KCN + 8 \( \mu \)M insulin | 12 |
| 0.5 \( \mu \)M N-ethylmaleimide | 8 |
| 0.5 \( \mu \)M N-ethylmaleimide + 8 \( \mu \)M insulin | 8 |

![Fig. 2. Rate of \(^{86}Rb^+\) uptake as a function of \( Rb^+ \) concentration, in the presence and absence of insulin. Fat cells were washed and resuspended in KCl-free buffer containing the indicated concentration of \( RbCl\). Each set of tubes contained 0.5 ml of a 30% adipocyte suspension and was incubated for 10 min in the absence (\( \square \)) or presence (\( \bullet \)) of 8 \( \mu \)M insulin. Transport was initiated by the addition of 2.5 \( \mu \)Ci of \(^{86}RbCl\) (final concentration, 60 \( \mu \)M). Ten minutes later, triplicate 100-\( \mu \)l aliquots were withdrawn and processed. Each point is the average of triplicate determinations.](image)

In the absence of ouabain, the rate of \(^{22}Na^+\) tracer equilibration was extremely rapid (less than 2 min), and the level of \(^{22}Na^+\) in the cell remained constant for at least 2% h (Fig. 4B). Using the data of Fig. 4B and a concomitant measure of net intracellular water volume, we calculated that the internal \( Na^+ \) concentration was approximately 15 \( m \) (\( \pm 5 \) S.D.). The amount of tracer in the insulin-treated cells was slightly lower, but this difference is not statistically significant. In contrast, in the presence of ouabain (Fig. 4B, upper curve), the \( Na^+ \) level rose to nearly 120 \( m \) by 3 h, suggesting that the \( Na^+ \) concentration gradient normally maintained by the \((Na^+,K^+)-ATPase\) had been dissipated.

The next set of experiments was designed to determine the effect of insulin on \((Na^+,K^+)-ATPase\)-mediated \( Na^+ \) efflux. When cells were preincubated with \(^{22}Na^+\) and resuspended in nonradioactive Krebs-Ringer buffer, no difference in the loss of \(^{22}Na^+\) from the cells was observed in the presence or absence of 1 \( mM \) ouabain (data not shown). One explanation for this observation is that a considerable portion of the isotope "loss" is due to the rapid exchange of internal radioactive \( Na^+ \) for external unlabeled \( Na^+ \). Accordingly, adipocytes were loaded with \(^{22}Na^+\) in Krebs-Ringer buffer and then resuspended in \( Na^-\)-free choline Ringer buffer (see under "Experimental Procedures") in order to monitor net isotope efflux. The results of this experiment are shown in Fig. 5. The half-time for \(^{22}Na^+\) efflux was 2 min; in the presence of 1 \( mM \) ouabain, the \( t_{1/2} \) efflux was 9 to 10 min.

Unexpectedly, no effect on the rate of ouabain-inhibitable \(^{22}Na^+\) efflux was detected when insulin was added. We then tested whether adipocytes remain insulin responsive under the experimental conditions used to minimize \(^{22}Na^+\) efflux. L-Arabinose is a nonmetabolizable sugar whose transport has been shown to occur through the D-glucose carrier and to be stimulated by insulin (25). When adipocytes were loaded with \(^{3}H\) arabinose in Krebs-Ringer buffer and then resuspended in choline Ringer buffer, no increase in arabinose efflux was observed upon addition of insulin (data not shown). However, cells incubated for an additional 20 min in choline
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Ringer buffer did exhibit insulin-stimulated arabinose flux. These observations agree with the results of Vega and Kono (26), who reported that mechanical agitation (such as shaking or centrifugation of the cells) transiently blunts the insulin effect on sugar transport in fat cells. Since it was not possible to resuspend cells in Na⁺-free buffer without centrifugation or shaking nor was it possible to allow the cells to "recover" for 15 min following centrifugation (at which time all the internal ²²Na⁺ would have been lost), an experiment to measure insulin-stimulated Na⁺ efflux was technically not feasible. This difficulty could be overcome if we could find an inhibitor of the ouabain-insensitive component of Na⁺ influx. To date, no inhibition of ²²Na⁺ uptake has been obtained with 0.5 mM amiloride, 1 μM tetrodotoxin, or 1 mM tetracaine.

Fig. 4. ²²Na⁺ uptake. A, effect of insulin on ²²Na⁺ entry; early time points. Two tubes, each containing 3.0 ml of 40% adipocytes, were incubated for 15 min at 37°C with 10⁻³ M ouabain. Ouabain was present to prevent efflux of Na⁺ through the (Na⁺,K⁺)-pump. At time zero, 30 μCl of ²²NaCl (carrier free) was added to both tubes, and one tube received 3 nM insulin. Aliquots (100 μl each) were withdrawn at the indicated times. ²²Na⁺ uptake is expressed as a percentage (²²Na⁺ counts per min in cells/total ²²Na⁺ counts per min) per 100-μl aliquot, after subtraction of trapped volume. Each data point represents the average of duplicate determinations (control; 3 nM insulin). Statistical analysis of individual real data points revealed no significant difference in uptake values (p > 0.10 by paired Student t test). B, ²²Na⁺ tracer equilibration levels. Adipocytes (2.0 ml, 30% cells) were incubated for 15 min at 37°C in the presence or absence of 8 nM insulin or 1 mM ouabain. At time zero, 30 μCl of ²²NaCl was added to each tube, and ²²Na⁺ uptake was determined as described above. Each data point represents the average of duplicate determinations. Symbols are the same as in Fig. 1.

Table II

| [NaCl] | Rate of ²⁶Rb⁺ uptake |
|--------|---------------------|
| mM     | Minus insulin | Plus insulin |
| 0      | 0.5          | 0.5          |
| 75     | 4.2          | 5.4          |
| 145    | 6.7          | 8.5          |
| 195    | 6.6          | 8.4          |

Effect of external NaCl on the rate of ²⁶Rb⁺ uptake

Fat cells were washed and resuspended in Ringer buffer containing the indicated concentrations of NaCl. The Na⁺ concentration was varied by iso-osmotic substitution of choline chloride for NaCl. The sodium-free buffer contained 10 mM Tris-phosphate, pH 7.4, instead of 10 mM sodium phosphate. Following a 15-min incubation at 37°C in the presence or absence of 8 mM insulin, 2 μCi/ml of ²⁶RbCl was added to all tubes. Duplicate 100-μl aliquots were withdrawn 5 and 10 min later. The ouabain-resistant rate has been subtracted from all values.

2 R. A. Nemenoff, unpublished results.
3 M. D. Resh, unpublished results.
centration was approximately 40% higher than in control untreated cells (data not shown).

The hypothesis that insulin activates \(^{32}\)Rb\(^+\) uptake by increasing Na\(^+\) entry is untenable in the rat adipocyte for the following reasons. No change in \(^{22}\)Na\(^+\) entry was detected in the presence of insulin (Fig. 4A). The basal rate of Rb\(^+\) uptake was not increased when the external Na\(^+\) concentration was raised from 145 mM to 195 mM, and the insulin-stimulated rate at both Na\(^+\) concentrations was identical (Table II). If insulin acted by increasing the membrane permeability to Na\(^+\) ions, we would have expected to see a much larger insulin stimulation of the rate of Rb\(^+\) uptake with a 13:1 Na\(^+\) concentration gradient (195 mM) compared to that observed at 145 mM Na\(^+\). Finally, although the Na\(^+\) ionophore monensin stimulates \(^{32}\)Rb\(^+\) uptake to the same extent as insulin, it does so by increasing the intracellular Na\(^+\) concentration. Clearly, no such increase in Na\(^+\) content was observed in the presence of insulin (Fig. 4B). Taken together, these results suggest that insulin acted to increase the activity of the (Na\(^+\),K\(^+\))-ATPase and that this effect was not due to an alteration of intrinsic membrane permeability to Na\(^+\) or K\(^+\), nor to the opening of an ion-specific gate or "channel" in the membrane (see under "Discussion").

Requirement for the Insulin Receptor—Several lines of evidence support the claim that stimulation of Rb\(^+\) uptake requires intact insulin and an intact insulin receptor. Insulin was briefly incubated at 100°C and then repeatedly frozen and thawed. This "heat-inactivated" insulin no longer stimulated Rb\(^+\) uptake, whereas native untreated hormone did. When epididymal fat pads were treated with high concentrations of insulin \((195 \text{ mM})\) during collagenase digestion, the cells isolated from this tissue exhibited a stimulated rate of Rb\(^+\) uptake. Subsequent addition of 3 nM insulin was without further effect.

The insulin concentration dependence of this stimulation is depicted in Fig. 6. The half-maximal effect occurred at 200 pM insulin, a concentration at which only 2% of the insulin-bind-

![Fig. 6. Dependence of stimulation of \(^{32}\)Rb\(^+\) uptake on insulin concentration. The basal rate of \(^{32}\)Rb\(^+\) uptake was calculated for the initial 10 min of the transport assay. The effect of various concentrations of insulin on the uptake rate is expressed as a percentage (stimulated rate - basal rate/basal rate). Each point represents the average of two to six separate rate determinations. Half-maximal stimulation occurs at approximately 200 pM insulin.](image)

![Fig. 7. Reversal of insulin stimulation by trypsinization. Four tubes, each containing 2.1 ml of 40% adipocytes, were incubated for 10 min at 37°C in the absence (A, ○) or presence (△, ○) of 3 nM insulin. All tubes then received 5 μCi of \(^{32}\)RbCl at time zero. Twenty minutes later, 1 mg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin was added to two tubes (●, ○). All data points represent the average of duplicate determinations. A, the basal rate of Rb\(^+\) uptake is unaffected by 1 mg/ml of trypsin. B, trypsin lowers insulin-stimulated Rb\(^+\) uptake to a rate approximating that of control cells.](image)
that the rat adipocyte contains at least two types of (Na',K')-ATPases. The insulin stimulation of (Na',K')-ATPase activity described above indicates that insulin stimulates the uptake of (Na',K')-ATPase activity in adipose tissue. The hormone effect was mediated by physiological concentrations of insulin, required insulin binding to its intact cell surface receptor, and was reversible by trypsinization.

Insulin activation of monovalent cation transport has been reported in several other systems (4-15). Exposure of rat soleus muscle (8, 32), duck salt gland (13), and rat uterine muscle (11) to 100 milliunits/ml of insulin (740 nm) results in 20 to 30% increases in ouabain-inhibitable 32K+ activity (or 86Rb+) influx. Relatively few studies have investigated hormone-sensitive potassium accumulation in adipose tissue. The K+ content of rat fat pads increases when the tissue is incubated for 3 h at 37°C in the presence of 50 milliunits/ml of insulin and 8 mM glucose, with no change evident in the level of Na+ (13). In isolated mouse fat cells, a decrease in 42K+ uptake induced by adenine is prevented when insulin is present; however, insulin alone does not alter net K+ uptake (14). Clausen et al. (33) reported that rat fat cell ghosts take up 42K+ by an energy-dependent ouabain-inhibitable process but did not investigate the effect of insulin. Prior to this report, therefore, no study had demonstrated a direct effect of insulin on K+ uptake in an intact, isolated cell preparation. Moreover, the insulin concentration dependence for this effect was not de-

**Fig. 8.** Scatchard plot of [3H]ouabain binding to intact fat cells. A 40% suspension of adipocytes was placed into tubes containing 4 nm insulin (○) or an equivalent volume of buffer (●) and incubated at 37°C for 10 min. [3H]Ouabain was added to a final concentration of 1 x 10^{-8} M, and nonradioactive ouabain was added to final concentrations of 5 x 10^{-8} M to 1 x 10^{-9} M. Following a 35-min incubation at 37°C (at which point binding equilibrium is achieved), duplicate 200-μl aliquots were withdrawn from all tubes. The concentration bound was calculated by (bound counts per min/total counts per min) x total concentration. No correction for "non-specific" binding sites could be made, since this would require concentrations of ouabain of at least 10 to 100 mM. Ouabain is insoluble in aqueous solution at these concentrations. B, bound; F, free.

**Fig. 9.** Ouabain inhibition of 86Rb+ uptake. Each tube contained 0.5 ml of 30% adipocytes and the indicated concentration of ouabain. Following a 25-min incubation at 37°C, half the tubes received 8 mM insulin (○) and the other half, an equivalent volume of buffer (●). Ten minutes later, 2.5 μCi of 86RbCl was added to all tubes. Triplicate 100-μl aliquots were withdrawn at time = 10 min. The rate was determined as counts per min in cells at 10 minutes minus trapped volume.

**DISCUSSION**

**Insulin Stimulation of K+ Transport**—The results presented here indicate that insulin stimulates the uptake of 86Rb+ mediated by the (Na',K')-ATPase in the rat adipocyte. Within 2 min after exposure to the hormone, a 50% increase in the rate of 86Rb+ uptake was observed in isolated fat cells. As a consequence, a higher level of intracellular potassium was achieved (basal = 180 mM; insulin-stimulated = 220 mM K+). The uptake of 86Rb+ in the presence and absence of insulin was activated by external K+ and external Na+, was energy dependent, and was ouabain inhibitible. These observations strongly suggest that insulin stimulates the (Na',K')-ATPase activity of the rat adipocyte. The fat cell contained at least two types of (Na',K')-ATPases, one of which was stimulated by insulin. Insulin acted by increasing the V_{max} of Rb+ entry into the cell, with no immediate effect on the exit of Rb+ or entry of Na+. The hormone effect was mediated by physiological concentrations of insulin, required insulin binding to its intact cell surface receptor, and was reversible by trypsinization.
determined in any tissue type previously investigated.

Stimulation of the (Na⁺,K⁺)-ATPase in quiescent mouse 3T3 cells by serum has been documented (34) and exhibits kinetic characteristics similar to those observed in rat adipocytes. Serum stimulates the Vₘₐₓ of Rb⁺ influx without affecting Rb⁺ efflux. The stimulation observed is rapid (within 2 min after addition of serum) and is blocked by ouabain (34). However, the primary effect of serum on 3T3 cells appears to be an increase in Na⁺ entry into the cells (29), which then activates the (Na⁺,K⁺) pump, resulting in an increase in Rb⁺ uptake. Although increasing the intracellular Na⁺ concentration (in the presence of monensin) will stimulate the uptake of Rb⁺ in adipocytes, this mechanism is probably not involved in the stimulation of fat cell (Na⁺,K⁺)-ATPase by insulin. When Na⁺ efflux through the pump was prevented by ouabain, no increase in the rate or amount of 22Na⁺ entering the adipocyte was detected in the presence of insulin (Fig. 4A). There was no significant change in the intracellular steady state Na⁺ level when insulin was present (Fig. 4B). Finally, increasing the Na⁺ concentration gradient (by altering the external NaCl concentration) did not magnify insulin stimulation of Rb⁺ uptake. Thus, although the activity of the adipocyte (Na⁺,K⁺)-ATPase was dependent on the external:internal Na⁺ concentration gradient and the intracellular Na⁺ concentration, it did not appear that the mechanism of insulin stimulation of the pump involved a noticeable alteration in the cell's internal level of sodium. Other reports that have documented increases in K⁺ content in rat uterus and fat pads have not observed any change in Na⁺ levels with insulin (11, 13).

In principle, it should be possible to detect a concomitant increase in Na⁺ efflux from a cell or tissue type in which insulin stimulates the (Na⁺,K⁺)-ATPase. Such increases in Na⁺ efflux have been reported for various muscle preparations (8, 10, 12). The rat adipocyte contains 15 fmol of Na⁺/cell (Fig. 4B). Assuming a pumping rate of 2 × 10⁶ ions/cell/min (Fig. 2), we calculate that it would take 4 to 5 min for a fat cell to pump out all the available internal Na⁺. This is what was found experimentally (Fig. 5). As mentioned under "Results," rapid resuspension of adipocytes in Na⁺-free buffer transiently obscured insulin stimulation of membrane transport events and, therefore, no hormone effect on net 22Na⁺ efflux could be observed.

It must be emphasized that accurate measurements of intracellular concentrations of Na⁺ are extremely difficult due to the miniscule intracellular water volume of the fat cell (about 10⁻¹⁵ liters/cell) (21). Although the amount of 22Na⁺ in the insulin-treated cells was slightly lower than that of control cells (Fig. 4B), the variance in the data makes it impossible to state that this difference is significant (p > 0.10 by the Student t test). In order to be considered statistically significant, the decrease in sodium content of insulin-treated cells would have had to be greater than 5 mM, when compared to the Na⁺ content of control cells (15 mM± ± S.D.).

The results presented in this paper indicate that insulin stimulated the activity of the adipocyte (Na⁺,K⁺)-ATPase. In the presence of hormone, the initial rate of uptake of K⁺ was increased by 50%, there was no change in K⁺ efflux, and as a consequence, the steady state intracellular K⁺ concentration was increased by 40 mM. We did not detect a decrease in the steady state level of intracellular Na⁺. One possible explanation for this observation is that anything less than a 30% decrease in internal Na⁺ content would have been obscured by the variance in the data (i.e. a change from 15 mM to 10 mM Na⁺ could not be considered significant). If the intracellular Na⁺ concentration was indeed lower in insulin-treated cells, then, assuming no change in the rate of Na⁺ influx (Fig. 4A), one would expect that the activity of the Na⁺ pump would fall until a new steady state was reached. At this point, rubidium entry and sodium efflux rates would approximate those rates observed before insulin was added. This hypothesis predicts that insulin stimulation of Rb⁺ entry would be observable only as a transient effect. However, we have observed increased rates of Rb⁺ influx in the presence of insulin for as long as 60 min. Since it is unlikely that it would take more than 5 min to readjust intracellular Na⁺ levels (Fig. 5) and thus the activity of the pump, the effect of insulin is probably to increase the steady state rate of Rb⁺ influx through the (Na⁺,K⁺)-ATPase.

Alternatively, insulin stimulation of the pump may effect a transient decrease in the internal Na⁺ concentration, which would increase the external:internal Na⁺ gradient. (For example, decreasing the internal Na⁺ concentration from 15 mM to 10 mM would increase the Na⁺ concentration gradient from 9.67:1 to 14.5:1, a 50% higher value.) Nonpump-mediated Na⁺ influx might then rapidly restore the original intracellular Na⁺ levels, and thus no change in the steady state concentration of Na⁺ would be detected. Thus, an increased rate of Na⁺ influx would be observed only in the presence of unstimulated active Na⁺ pump. Given the resolution of current methods employed to monitor Na⁺ transport, we would not be able to detect a rapid transient effect of insulin on 22Na⁺ fluxes.

There is an additional complication concerning the observation that during stimulation by insulin the K⁺ (Rb⁺) concentration in the fat cell increased while there was not a measurable similar decrease in the Na⁺ concentration. This new distribution of ions requires that either the volume of the fat cell increases, or that there is sequestration of K⁺ ions into intracellular compartments, or that there is excretion of some other solute to decrease the osmolarity of the cell, or that the cell membrane is not permeable to water. Since the volume of the fat cell did not change after exposure to insulin (control, 0.86 picoliter/cell ± 0.08, S D; insulin treated, 0.83 picoliter/cell ± 0.07, S.D.), and the possibility that the membrane is not permeable to water is unlikely, one must presume that osmotic balance is achieved either by intracellular sequestration of K⁺ ions or by loss of some unidentified solute.

Ouabain-binding Sites—Ouabain is a specific inhibitor of the (Na⁺,K⁺)-ATPase. The affinity of the pump for the cardiac glycoside depends on the tissue type and the nature of the ligands present (35). The (Na⁺,K⁺)-ATPase of rat tissues is relatively insensitive to ouabain; its affinity for this cardiac glycoside is 10⁻⁵ to 10⁻⁴-fold lower than that of the enzymes of other organisms (36, 37). In agreement with the data obtained from other rat tissues, complete inhibition of rat adipocyte ATPase was not achieved until the ouabain concentration reached 10⁻² M. However, a more detailed kinetic analysis revealed a subset of pumps with high affinity for ouabain (Fig. 9). The biphasic nature of the ouabain inhibition curve implies that, in the adipocyte, at least two different pump sites are actively pumping Rb⁺, with Kᵢ values for ouabain of 1 × 10⁻⁴ M and 1 × 10⁻³ M, respectively. A recent publication from this laboratory documents the existence of two distinct molecular forms of the (Na⁺,K⁺)-ATPase in brain tissue (38), with Kᵢ values for the inhibition of ATPase activity by strophanthinid of 2 × 10⁻³ M and 2 × 10⁻⁴ M. These two forms are localized in different subfractions of nervous tissue. The two (Na⁺,K⁺)-ATPases described in this paper appear to be in the same cell type. The procedure utilized for separation of cells from medium selects only for those cells which are of density less than that of the oil layer (specific gravity 0.98 (21)); any contaminating cell types would pellet under these conditions. We conclude that the rat adipocyte contains two types of
(Na⁺,K⁺)-ATPase which can be distinguished by their different affinities for ouabain and sensitivity to insulin. The Scatchard plot of ouabain binding to intact adipocytes [Fig. 9] is nonlinear, consistent with the existence of more than one class of ouabain-binding site. The area of interest is the high affinity portion of the curve, since this corresponds to the class of insulin-stimulated pump sites. These sites (Na⁺,K⁺)-ATPase which can be distinguished by their differences in number for ATP of 106/s (41) and that 2 K⁺ ions are transported for each ATP molecule hydrolyzed (42), we estimate that there are 2 × 10⁶ enzyme molecules per cell that are actively pumping K⁺. This means that less than 10% of the high affinity ouabain-binding sites are active pump sites. If there is a change in the number of active pumps in the presence of insulin, it will be obscured by the large number of inactive binding sites, and thus the change will be impossible to detect by Scatchard analysis. On the other hand, it is also possible that the rat (Na⁺,K⁺)-ATPase has a much lower turnover number than the enzyme from other organisms or that the adipocyte ion pumps are operating at 10% of the maximal rate. Whether insulin increases the number of (Na⁺,K⁺) pumps or increases the turnover number of the already active pumps remains an unresolved question.

**Insulin Stimulation of Membrane Transport Activity**—The most widely characterized effect of insulin on the fat cell is the stimulation of glucose transport (16–18). It is of interest to compare this process with the stimulation of Rb⁺ transport described in this paper. Both effects occur on a similar time scale for activation and require only 2 to 5% occupancy of the insulin receptors for half-maximal stimulation (19, 43). In addition, both effects require insulin binding to an intact cell surface receptor (30) but can be mimicked by addition of anti-adipocyte membrane antibodies²,³,⁴ (44). However, the stimulation of Rb⁺ uptake occurs in the absence of glucose in the medium and is not inhibited by cytochalasin B, a specific inhibitor of basal and insulin-stimulated glucose uptake. The presence of 10⁻⁴ M ouabain does not immediately affect basal or insulin-stimulated hexose uptake in adipocytes or soleus muscle (45), although prolonged exposure (90 min) to ouabain has been reported to stimulate 3-O-methylglucose efflux in muscle (45). L-Arabinose transport is unaffected when adipocytes are suspended in Na⁺-free medium;² but basal and insulin-stimulated Rb⁺ uptake rates are completely inhibited under these conditions. Clearly, uptakes of monovalent cations and sugars are mediated by two separate membrane transport systems. Given the similarity in the way that insulin affects these two transport systems, it is reasonable to speculate that hormone interaction with the insulin receptor generates a common signal which serves to activate two different membrane transport proteins. The nature of this putative coupling mechanism is currently under investigation.

**Acknowledgments**—We wish to thank Lew Cantley for many discussions.

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² Anti-adipocyte membrane antiserum was a kind gift from Dennis Pilion, Brown University, Providence, RI.

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