The Metabolism of Isolated Fat Cells

VII. SODIUM-LINKED, ENERGY-DEPENDENT, AND OUABAIN-SENSITIVE POTASSIUM ACCUMULATION IN GHOSTS*

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SUMMARY

A method is described for the study of transport processes in ghosts isolated from rat adipose cells. With this procedure it was possible to follow the time course of potassium uptake, potassium release, and sodium release. The uptake of potassium was stimulated by sodium and was inhibited by ouabain at concentrations down to at least 10⁻⁶ M. In the presence of pyruvate (1 mm), potassium uptake showed a more rapid initial increase, and the steady state level was more than doubled. Glucose (5 mm) produced a smaller stimulation of potassium uptake. Cyanide, 2,4-dinitrophenol, or incubation in the cold diminished both potassium uptake and content. Potassium efflux, unlike uptake, was not altered by ouabain or pyruvate; a half-life of 45 min was found.

Sodium ion was exchanged but not accumulated by ghosts. Efflux studies showed that sodium was released with a half-life of 10 min.

It is concluded that potassium is accumulated by ghosts of fat cells via a sodium-linked, energy-requiring process characteristic of an intact plasma membrane.

It is well established that fat cells, isolated from rat adipose tissue, are extremely sensitive to the actions of insulin and several lipolytic hormones (1). As it seems possible that the plasma membrane is the initial site of action of these hormones (2), our efforts were logically directed toward obtaining information about those processes in the plasma membrane that may be affected, directly or indirectly, by the actions of hormones on the membrane. In order to study direct effects of hormones on the plasma membrane, it was considered essential to separate, as much as possible, the plasma membrane from those processes or components within the cell that have the potential, when secondarily affected by the hormones, of influencing events occurring at the membrane. To a large extent this requirement has been met by the isolation of ghosts of fat cells (3), which have been described as sacs of plasma membrane that are semi-permeable to ions and within which are enclosed 26% of the mitochondria, endoplasmic reticulum, and about 2% of the soluble enzymes present in fat cells. About 20% of the ghosts are nucleated, but all are essentially devoid of fat. The plasma membrane of ghosts contains a carrier-mediated glucose transport system which is stimulated by insulin (4) and adenylyl cyclase which is stimulated by adrenocorticotropic, glucagon, and epinephrine (2, 3). The latter findings show that ghosts are sensitive to hormones and that the plasma membrane contains at least two systems that are regulated by hormones.

Active coupled sodium-potassium transport, sometimes referred to as the sodium-potassium pump (5), and amino acid transport are other processes in the plasma membrane of animal cells that may be regulated by hormones. In this and an accompanying report (6), some of the basic characteristics of these transport processes have been examined in fat cell ghosts with the purposes both of providing more evidence that the hormonally sensitive plasma membrane of ghosts (or fat cells) contains most of the functional components that are characteristic of other animal cells, and of stimulating other investigators to use this material for exploring various aspects of the regulation of membrane transport systems by hormones.

EXPERIMENTAL PROCEDURE

Male Wistar rats (150 to 200 g) were used in this study. They had free access to Altromin R laboratory chow (Kunath Company, Aarau, Switzerland).

Reagents and Enzymes—Human serum albumin was purchased from the Central Laboratory of the Swiss Red Cross, Berne, and used after dialysis for 24 hours against distilled water. ATP (disodium salt) was obtained from Boehringer, ouabain from Calbiochem, and bacterial collagenase from Worthington. All chemicals used were of analytical grade. *Na and *K (produced by irradiation of the carbonates and subsequent
neutralization with HCl, specific activity 100 mCi per g) were obtained as aqueous solutions of the chlorides from two sources: Eidg. Institut für Reaktorforschung, Würenlingen, Switzerland, or Isotope Division, Research Establishment Risø, Roskilde, Denmark. Uniformly labeled sucrose-\(^{14}C\) and inulin-\(^{3}H\) were products of New England Nuclear.

**Preparation of Fat Cell Ghosts**—Fat cells were isolated from epididymal fat pads and ghosts were prepared essentially as described earlier (1, 3). In the present study the hypotonic medium used for lysis of fat cells had the following composition: Na\(_2\)ATP, 2.5 mM; NAD, 0.1 mM; MgCl\(_2\), 2.5 mM; CaCl\(_2\), 0.1 mM; KHC\(_2\)O\(_3\), 1.0 mM. The pH was adjusted to 7.2 with Tris.

Ghosts were isolated by centrifugation for 10 min at 900 \(\times g\) in the cold (3). The sediment was resuspended in the hypotonic medium and sucrose was added to give a final concentration of 300 mM. This suspension was centrifuged for 10 min at 900 \(\times g\), the supernatant fluid was discarded, and the sediment was suspended in ice-cold incubation medium. Ghosts could be kept at 0° for as long as 20 min without altering the potassium uptake as subsequently measured at 37° (see Fig. 5).

**Incubation Conditions**—Most incubations took place at 37° in a metabolic shaker (80 cpm). Incubations were carried out in polyethylene vials. Initial incubation volume varied between 1 and 3 ml, and the content of ghosts corresponded to 0.1 to 0.25 mg of protein per ml. Unless stated otherwise, Krebs-Ringer bicarbonate buffer (7), pH 7.4, containing 1% albumin was used as the basic incubation medium. The incubation vials were gassed with a mixture containing 95% \(O_2\) and 5% \(CO_2\).

**Influx Experiments**—In studies of \(^{42}K\) uptake, ghosts were suspended in ice-cold incubation medium modified to contain 1 mmole of potassium. Aliquots of this suspension were distributed into incubation vials containing a medium of the same composition. After incubation for 10 min at 37°, \(^{42}K\) was added together with an amount of unlabeled KCl sufficient to give a final concentration of 6 mm potassium in the incubation mixture. In \(^{24}Na\) uptake studies, radioactive sodium ion was added to suspensions of ghosts that had been incubated for 10 min at 37° in Krebs-Ringer albumin medium. The moment of addition of radioactive ions is referred to as zero time.

For sampling, duplicate 0.2-ml aliquots of the incubation suspension were layered over 0.1 ml of chilled unlabeled medium contained in plastic micro-test tubes (Beckman Catalogue 314326). The tubes were immediately centrifuged for 30 sec at 10,000 \(\times g\) in a Beckman microcentrifuge (Catalogue 314300). The supernatant fluid was aspirated with a blunted No. 10 cannula, 0.2 ml of ice-cold unlabeled medium was added without disturbing the pellet, and the tube was centrifuged for 15 sec at 10,000 \(\times g\). The tip of the tube was cut off just above the pellet with a scalpel blade and transferred to a counting vial containing 10 ml of water. The pellet was dispersed in the water with a Vortex shaker.

Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer, model 3002. The \(\beta\)-particles emitted by \(^{42}K\) and \(^{24}Na\) are fast enough to allow detection by the Cerenkov radiation produced in water (8). Aliquots of the incubation medium were counted also in 10 ml of water.

The specific activity of \(^{42}K\) in the incubation medium was calculated from the potassium content as measured by flame photometry. On this basis the radioactivity in the pellets was converted to nanomoles of potassium taken up. It is probable that the ghosts, at the moment of \(^{42}K\) addition, contain unlabeled potassium which could exchange during incubation with \(^{42}K\) and diminish the “extracellular” specific activity. However, as the concentration of ghosts per ml of incubation mixture ranged between what corresponds to 0.1 to 0.25 mg of protein, it was calculated, assuming the ghosts to contain an unlikely concentration of 150 nm potassium, that the amount of unlabeled potassium which could possibly be released from the ghosts never exceeded 0.1 \(\mu\)mole per ml of incubation medium. As this amount would only change the specific activity by less than 2%, measurements of \(^{42}K\) in the pellets gave a reasonable estimate of the amount of potassium taken up by ghosts during incubation. The operational terms “\(^{42}K\) content” (zero time activity included) and “\(^{42}K\) uptake” (zero time activity subtracted), calculated as micromoles of potassium, are used as expressions of potassium uptake by ghosts.

In each experiment, representative samples of the incubation mixture were withdrawn in duplicate and pellets of ghosts isolated (with 0.154 m NaCl for washing) for determination of the protein content by the method of Lowry et al. (9). Influx Experiments—Loading of ghosts with \(^{42}K\) or \(^{24}Na\) was achieved by incubation for 30 min in medium containing the
Fig. 1. Effect of ouabain and cyanide on $^{42}$K uptake in fat cell ghosts

Fat cell ghosts were suspended in Krebs-Ringer bicarbonate buffer containing 1% albumin and the indicated additions. After 10 min of incubation $^{42}$K was added. Results are given as the increase in $^{42}$K content of ghosts after 15 and 60 min of incubation, determined in duplicate.

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<th>$^{42}$K content</th>
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<tr>
<td></td>
<td>15 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Control</td>
<td>108.2</td>
<td>175.1</td>
</tr>
<tr>
<td>Ouabain $10^{-6}$</td>
<td>82.1</td>
<td>118.4</td>
</tr>
<tr>
<td>Ouabain $10^{-5}$</td>
<td>63.7</td>
<td>116.3</td>
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<tr>
<td>Ouabain $10^{-4}$</td>
<td>40.2</td>
<td>65.1</td>
</tr>
<tr>
<td>Ouabain $10^{-3}$</td>
<td>29.8</td>
<td>55.0</td>
</tr>
<tr>
<td>Cyanide ($10^{-4}$)</td>
<td>32.8</td>
<td>30.1</td>
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The data shown in Fig. 1 show that ghosts take up $^{42}$K upon incubation for 1 hour and that uptake is proportional to the amount of protein in the pellet obtained by centrifuging the suspension of ghosts at $10,000 \times g$ for 30 sec. The zero time values show the efficiency of the washing procedure. The fact that $^{42}$K content at zero time increased with increasing amounts of material reflect a combination of (a) the amount of $^{42}$K taken up by ghosts during the 2-min interval required for sampling and washing, (b) trapped extracellular $^{42}$K, and (c) $^{42}$K bound but not transported.

The time course of $^{42}$K uptake, shown in Fig. 2, consisted of three successive phases: (a) a rapid uptake for the first 30 min, during which about 80% of maximal uptake was achieved; (b) a gradual uptake until at 60 min the maximal uptake was attained, which probably represents the steady state concentration; and (c) a slight decline during the subsequent 60 min of incubation. It can also be seen in Fig. 2 that the time course of uptake showed little variation among replicate vials during the first 60 min of incubation.

It seems likely that the steady state phase represents the concentrating capacity of ghosts. This is supported by the finding that the addition of ouabain, an inhibitor of sodium-potassium transport (5), at the moment when maximal uptake was attained induced a subsequent decrease in the $^{42}$K content (Fig. 2). Furthermore, maintenance of the steady state level required energy since both dinitrophenol and cyanide caused a marked decline in $^{42}$K content.

In addition to their effects on the steady state levels of $^{42}$K, ouabain, at concentrations down to at least $10^{-6}$ M, and cyanide (10$^{-3}$ M) also inhibited the initial phase of $^{42}$K uptake (Table I). $^{42}$K uptake was dependent on the external concentration of potassium and was inhibited by ouabain at each concentration of potassium tested (Fig. 3). Uptake was progressively increased with increasing amounts of sodium in the medium (Fig. 3).
In the latter experiments, sodium chloride was replaced iso-osmotically with choline chloride, and Tris (pH 7.4) was substituted iso-osmotically for the sodium contributed by NaHCO₃ in the normal Krebs-Ringer bicarbonate buffer. Other experiments in which NaCl was replaced by LiCl gave similar results.

The above results show that ⁴²K accumulation was sensitive to ouabain, was stimulated by sodium, and was energy-dependent as reflected by the effects of metabolic poisons. In other experiments, it was found that incubation at ⁰°C nearly prevented the uptake of ⁴²K. These observations suggested that potassium uptake and maintenance of a concentration gradient may be substrate-dependent, a characteristic of an active process. Evidence in support of this is shown in Fig. 5 which describes the effects of substrates on ⁴²K uptake. Pyruvate (1 mM) had a striking effect, causing a 2-fold increase in both the rate of uptake and in the steady state level of ⁴²K. In other experiments it was found that as little as 0.1 mM pyruvate stimulated ⁴²K uptake; maximal effects were obtained with 1 mM pyruvate. Glucose also increased the uptake of ⁴²K, although to a lesser extent than pyruvate. Externally supplied ATP (2.5 mM) did not stimulate ⁴²K uptake.

When the uptake of ⁴⁰Na was determined in ghosts, measurable quantities of radioactivity were detected in pellets at zero time. However, in contrast to ⁴²K, this level did not change at any time of incubation up to a period of 60 min. It is likely that the ⁴⁰Na activity measured in the packed ghosts is comprised of the same components discussed above for ⁴²K activity in the pellet at zero time, i.e. extracellular, bound, and transported. That some of the ⁴⁰Na activity present at zero time reflects bound or transported ⁴⁰Na is suggested by the fact that it was possible to load the ghosts previously with ⁴⁰Na and to measure its release into unlabeled medium. Thus the ghosts were found to retain ⁴⁰Na for a period of time appreciably longer than one would expect if all of the ⁴⁰Na were extracellular (Fig. 6A). The time course of ⁴⁰K efflux is shown in Fig. 6B. Parenthetically it should be noted that, in both ⁴⁰Na and ⁴²K efflux studies (Fig. 6, A and B), the sum of the radioactivity recovered in the pellets and supernatant fluids gave a nearly horizontal line, which shows the efficacy of the methods used for sampling and counting. With the values of ⁴⁰Na activity measured in the pellets during the first 15 min of efflux, the half-life of the ⁴⁰Na retained in the ghosts was calculated to be about 10 min. In various experiments, similar calculations for the half-life of ⁴²K retained in the ghosts gave values between 42 and 47 min. It could be shown that the ⁴²K content of the pellets of ghosts, when plotted semilogarithmically, decreased rectilinearly with time. The presence of ouabain (1 mM) and pyruvate (1 mM) in the efflux medium did not affect the rate of ⁴²K efflux.

Since all of the results indicated that ⁴²K was accumulated in the ghosts, it seemed desirable to measure the actual concentration of "intracellular" potassium. Attempts were made,
The ability to accumulate potassium and extrude sodium against a concentration gradient is a basic characteristic of animal cells. It is well established that the active coupled transport of sodium and potassium is ouabain-sensitive and energy-dependent (5), the energy component being ATP derived from internal energy production, as shown with erythrocyte ghosts. The recent report (15) of a ouabain-sensitive sodium-potassium pump in ghosts (11, 12). Furthermore, this process is inhibited when either potassium or sodium ions in the extracellular medium are replaced by other cations (13, 14). All of these properties contribute to a portion of the extracellular water space, may help to resolve the question of how much potassium is concentrated by ghosts during incubation.

**DISCUSSION**

Although it has not been possible as yet to measure accurately the concentration of potassium in ghosts, the finding that ouabain, when present in a concentration range known to inhibit specifically the potassium accumulation in intact cells (15), diminished both the initial rate of $^{42}$K uptake and the magnitude of the steady state level attained, while not influencing the rate of $^{42}$K efflux, provides indirect but strong evidence that the $^{42}$K content at steady state reflects an accumulation of potassium against a concentration gradient. This conclusion is further supported by the energy requirements for $^{42}$K uptake. Indeed, stimulation of $^{42}$K uptake by pyruvate seems explicable solely as an effect on an accumulative process.

In summary, ghosts of fat cells accumulate potassium by a sodium- and energy-dependent process and thereby display another property characteristic of an intact plasma membrane. The method described in this report allows a semiquantitative evaluation not only of the time course of influx and efflux, but also of the potassium content under steady state conditions. These indices may reflect the functional state of the ghosts under various metabolic conditions or in the presence of hormones which have been shown to affect glucose transport or metabolism in this material (4). The object of current studies, some of which are reported in the accompanying paper on amino acid transport (6), is to determine whether such relationships exist in ghosts.

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