Insulin Affects the Sodium Affinity of the Rat Adipocyte (Na⁺,K⁺)-ATPase*

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The Kₐ₅₀ for intracellular sodium of the two forms of (Na⁺,K⁺)-ATPase which exist in rat adipocytes (Lytton, J., Lin, J. C., and Guidotti, G. (1985) J. Biol. Chem. 260, 1177–1184) has been determined by incubating the cells in the absence of potassium in buffers of varying sodium concentration; these conditions shut off the Na⁺ pump and allow sodium to equilibrate into the cell. The activity of Na⁺,K⁺-ATPase was then monitored with ⁴²Rb⁺/K⁺ pumping which was initiated by adding isotope and KC1 to 5 mM, followed by a 3-min uptake period. Atomic absorption and ⁰²Na⁺ tracer equilibration were used to determine the actual intracellular [Na⁺] under the different conditions. The Kₐ₅₀ values thus obtained were 17 mM for α and 52 mM for α(+). Insulin treatment of rat adipocytes had no effect on the intracellular [Na⁺] nor on the Vₘₐₓ of ⁴²Rb⁺/K⁺ pumping, but did produce a shift in the sodium ion Kₐ₅₀ values to 14 mM for α (p < 0.025 versus control) and 33 mM for α(+)(p < 0.005 versus control). This change in affinity can explain the selective stimulation of α(+) by insulin under normal incubation conditions.

Measurement of the Kₐ₅₀ for sodium ion of (Na⁺,K⁺)-ATPase in membranes isolated from adipocytes revealed only a single component of activation with a low Kₐ₅₀ of 3.5 or 12 mM in the presence of 10 or 100 mM KC1, respectively. Insulin treatment of the isolated membranes or of the cells prior to membrane separation had no effect on these values.

The ionic gradients of both sodium and potassium which exist across the plasma membrane of almost every eukaryotic cell are essential for volume and osmotic regulation are maintained by the (sodium and potassium ion)-activated ATP phosphohydrolase (EC 3.6.1.3). While there is a large body of knowledge concerning the structure and function of this enzyme which has been the subject of numerous recent reviews (Forgac and Chin, 1985; Jørgensen, 1982; Cantley, 1981), its regulation by a number of effector molecules, such as catecholamines and polypeptide hormones, is just beginning to be understood. Agents which are known to be mitogenic for cells [Na⁺,K⁺]-ATPase...
All the isotopes used (\[^{85}\text{Rb}Cl\], \[^{22}\text{NaCl}\], 2-deoxy-[\(^{14}\text{C}\)]glucose, [\(^{3}H\)]H\(_2\)O, [\(^{14}\text{C}\)]sucrose, and [\(^{14}\text{C}\)]urea) and also Aquasol were from New England Nuclear.

The ingredients of the coupled assay for ATPase activity (Tris-ATP, NADH, lactate dehydrogenase, and pyruvate kinase) were obtained from Sigma. Ouabain was purchased from Boehringer Mannheim, and Sigma. Acetonox was obtained from American Scientific Products.

Adipocyte Preparation—Adipocytes were obtained from 125-150-g rats essentially as described by Resh et al. (1980). Where the incubation buffer differed from the normal Krebs-Ringer phosphate albumin-supplemented one (KRBA buffer), the cells were first washed 3–4 times in the appropriate buffer before the incubation commenced.

Media without KCl containing various NaCl concentrations, always osmotically balanced with choline chloride, were prepared by mixing appropriate volumes of two solutions: one containing 150 mM NaCl, the other 150 mM NaCl and 150 mM KCl, both in a buffer composed of 2.5 mM Tris-phosphate, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), 4% (w/v) bovine serum albumin, pH adjusted to 7.4 with Tris base.

Water Space Calculation—This was determined using either the pair of [\(^{3}H\)]sucrose and [\(^{14}\text{C}\)]urea or [\(^{3}H\)]H\(_2\)O and [\(^{14}\text{C}\)]urea (3 mM and 6 pCi/ml). After either 2 min (H\(_2\)O/sucrose) or 10 min (urea/sucrose) to the isotopes were added together from a stock solution to give final concentrations of [\(^{3}H\)]H\(_2\)O (15 pCi/ml) and [\(^{85}\text{RbCl}\] (1–2 \(\mu\)Ci/tube). In some experiments 2-deoxy-[\(^{14}\text{C}\)]glucose (1–2 \(\mu\)Ci/tube, final concentration of 0.1 mM) was included in the preincubation in order to monitor the effect of the incubation conditions on the basal and insulin-stimulated levels of glucose transport. Quenching was accomplished as described above for the water space calculation. "NaCl" uptake was linear for at least 5 min under all conditions used in these experiments (data not shown).

When present, insulin was added 20 min into the preincubation at a final concentration of 0.15 pM.

Na\(^+\), K\(^+\) Uptake—Isolated adipocytes were preincubated at 7.5% (v/v) cell suspension in a total volume of 0.67 ml/tube for 30-60 min at 37 °C in the appropriate buffer supplemented with or without the indicated concentrations of ouabain. At the conclusion of the preincubation period, isotope uptake was initiated by the addition of 25 μl of a stock solution containing KCl (to give a final concentration of 5 mM) and [\(^{85}\text{RbCl}\] (1–2 \(\mu\)Ci/tube). In some experiments 2-deoxy-[\(^{14}\text{C}\)]glucose (1–2 \(\mu\)Ci/tube, final concentration of 0.1 mM) was included in the preincubation in order to monitor the effect of the incubation conditions on the basal and insulin-stimulated levels of glucose transport. Transport was quenched after 3 min by spinning duplicate 200-μl aliquots through dialylyphate as described by Resh et al. (1980). Uptake of [\(^{85}\text{Rb}\]/K\(^+\) was linear for at least 5 min under all conditions used in these experiments (data not shown).

In general, the best fit of the data was to a model with partial cooperativity between sodium-binding sites. For simplicity, all of the figures show curves which have been fitted using Equation 2. The \(K_{\text{Na}}\) values were obtained from the fits to Equation 2.

This analysis is in no way intended to imply any conclusions regarding mechanistic models for interaction of sodium with (Na\(^+\), K\(^+\))-ATPase. While the ouabain-inhibitable fraction of total ATPase activity of kidney microsomes was >95% and that of brain axolemma >85%, only about 10% of the bovine membrane ATPase could be inhibited with up to 5 mM ouabain. It was thus important to be able to subtract the non-ouabain-inhibitable component so the kinetics of sodium dependence could be determined. To do this, membranes were mixed with 2.5 ml of assay buffer containing the appropriate ionic composition, and then two 1.0-ml portions were added to matched cuvettes. The reference cuvette contained 5 μl of ouabain dissolved in dimethyl sulfoxide at 0.5 M, enough to give a final concentration of 2.5 mM; the sample cuvette contained an equal volume of dimethyl sulfoxide alone. The resulting activity was >85% dependent upon the presence of both sodium and potassium, further confirmation that it indeed represented true (Na\(^+\), K\(^+\))-ATPase activity.

RESULTS

In order to determine the \(K_{\text{Na}}\) for intracellular sodium it is necessary to modulate its concentration over a fairly wide range. Normally the Na\(^+\) pump of the rat adipocyte extrudes sodium from the cell at such a rate that the steady-state level of Na\(^+\) within the adipocyte is maintained at about 15 mM (Resh et al., 1980). The approach that was adopted here was to incubate the cells in a 0 mM K\(^+\) environment such that the Na\(^+\) pump, which requires extracellular K\(^+\), was shut down. Under these conditions, Na\(^+\) was no longer pumped out of the cell, and thus the intracellular Na\(^+\) equilibrated toward the concentration found outside the cell. By substituting a fraction of the extracellular NaCl with an equimolar amount of choline chloride to maintain osmolarity, differing intracellular Na\(^+\) levels could be generated. Preliminary data (not shown) and the results of Resh et al. (1980) suggested that sodium had reached levels close to equilibrium within an incubation period of 30–45 min. Thus the protocol chosen was to incubate the adipocytes at a low cell density (so leaking intracellular K\(^+\) would not lead to a significant level of K\(^+\) in the medium) for 30–60 min in media of varying Na\(^+\). At this time, the activity of the Na\(^+\) pump was monitored by adding KCl back to 5 mM simultaneous with tracer [\(^{85}\text{RbCl}\] and measuring uptake over a 3-min period. The relative contributions of the two forms of the Na\(^+\) pump to Ra+/K\(^+\) uptake were determined by employing their difference in affinity for ouabain.
(Lytton et al., 1985), a specific inhibitor of (Na⁺,K⁺)-ATPase. Thus transport was determined under three conditions: none, 3–10 μM, or 1 mM ouabain. This corresponded to no inhibition, inhibition of only α(+), and complete inhibition of both α and α(+) pumping. Simple subtraction revealed the activity of each form.

The results of such an experiment are shown in Fig. 1. It is clear that at low extracellular Na⁺ concentrations the activity of the α form of the enzyme constituted the majority of αRb+/K⁺ pumping. However, as [Na⁺] was raised, so the pumping component attributable to α(+) increased, until it reached a plateau with an activity about three times the maximum for α. These maximal pumping activities represented 90–100% of the maximum capacity of the (Na⁺,K⁺)-ATPase to hydrolyze ATP, as measured in membranes (see “Discussion” and Lytton et al. (1985) for a discussion of this approach and the validity of the assumptions). The observation that the ratio of maximal αRb+/K⁺ pumping of α(+) to α was the same as the ratio of number of molecules of the two enzymes found in adipocyte membranes (Lytton et al., 1985) is consistent with the idea that the two enzymes are pumping at a rate limited by their identical maximum turnover numbers. Table I summarizes these calculations.

In order to compare the Kₐ₅ values of the two forms of the pump for Na⁺, the actual intracellular [Na⁺] under the different incubation conditions was determined. These measurements were made using both 22Na tracer equilibration and atomic absorption spectroscopy and are shown in Table II. Also included in the table are the concentrations of K⁺ within the cell and the intracellular volumes measured under all the conditions used. Since the volume of the cells remained approximately constant under all incubation conditions, one presumes the balance of the ionic species necessary to maintain isomolarity with the external milieu was contributed by choline chloride, which must have crossed the membrane readily. By interpolation of the correlation between intra- and extracellular [Na⁺], Kₐ₅ values for the α and α(+) forms of the Na⁺ pump for intracellular sodium ion in the resting state were estimated as 17 and 52 mM, respectively.

Having established a satisfactory protocol for measuring these constants, it was then essential to demonstrate that under the conditions of incubation used, the adipocytes remained insulin responsive. This was achieved by measuring the influence of insulin treatment on 2-deoxyglucose uptake. The cells responded with a 5-fold stimulation of uptake in normal KRPA, while under the conditions of 0 mM KCl and varying NaCl, a range of 3–5-fold stimulation was observed (data not shown). Table II also demonstrates that the short insulin treatment used (10–20 min) led to neither a significant change in intracellular Na⁺ or K⁺ concentrations nor was the intracellular volume affected under these conditions. Had the incubation period been continued for longer, one would have anticipated a rise in intracellular [K⁺] as observed by Resh et al. (1980).

The influence of insulin upon αRb+/K⁺ pumping under differing Na⁺ concentrations is shown in Fig. 2. While there was no apparent change in the Vₐ₉₅ of pumping, there was a substantial change in the affinity for Na⁺ ion. After correcting for the true intracellular concentration of Na⁺, insulin resulted in a shift in the Kₐ₅ values from 17 to 14 mM for α and from 52 to 33 mM for α(+) and a smaller shift in the α Kₐ₅. A paired t-test confirmed the significance of these changes, p < 0.005 for α(+) and p < 0.025 for α.

To test whether the different Kₐ₅ values for sodium and the

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**Fig. 1. Sodium dependence of αRb+/K⁺ pumping in adipocytes.** Suspensions of adipocytes (7.5%) were preincubated in modified KRPA buffer without KCl, including the indicated concentration of sodium ([NaCl] + [choline Cl] = 150 mM) and either none, 3–10 μM, or 1 mM ouabain for 30–60 min at 37 ºC. αRb+/K⁺ uptake was then determined over 3 min by simultaneously adding isotope and KCl to 5 mM. For further details see “Experimental Procedures.” ○, α activity = (uptake at 3–10 μM ouabain) – (uptake at 1 mM ouabain); Δ, α(+) activity = (uptake without ouabain) – (uptake at 3–10 μM ouabain). A, the data are the average of 2 experiments and are expressed as nmol of αRb+/K⁺ taken up/min/°C × 10⁶ cells. B, the same data as in A have been replotted as a per cent of their maxima, which were 1.8 and 6.0 nmol/min/°C × 10⁶ cells for α and α(+), respectively. The curves represent the best fit of the data to a model where 3 sodium ions are required for activity. The Kₐ₅ values are 15 mM for α and 86 mM for α(+).

**Fig. 2.** The influence of insulin upon αRb+/K⁺ pumping under differing Na⁺ concentrations. The curves represent the best fit of the data to a model where 3 sodium ions are required for activity. The Kₐ₅ values are 15 mM for α and 86 mM for α(+).
The combined data from 8 experiments are shown. The presence of 100 mM KCl, this affinity ATPase of adipocyte membranes is about 75% of the maximum affinity (a(+) = 12 mM). Pretreatment of the cells with insulin prior to incubation of the isolated membranes or membrane isolation or incubation of the isolated membranes appears that whatever regulates the affinity of the Na+ pump in rat adipocytes has quite different forms of the Na+ pump in rat adipocytes have quite different affinities for sodium ion in the whole cell. While a has a K0.5 for sodium ions required for activity. The K0.5 values are: panel A, control (a), 22 mM; insulin (a), 15 mM; control (a(+)), 85 mM; insulin (a(+)), 55 mM; panel B, control (a), 17 mM; insulin (a), 14 mM; control (a(+)), 52 mM; insulin (a(+)), 33 mM.

**TABLE II**

<table>
<thead>
<tr>
<th>Conditions of incubation</th>
<th>Volume*</th>
<th>[Na+]'</th>
<th>[K+]’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>picoiters/cell</strong></td>
<td><strong>mM</strong></td>
<td><strong>mM</strong></td>
<td></td>
</tr>
<tr>
<td>Normal KRPA</td>
<td>2.1</td>
<td>17</td>
<td>154</td>
</tr>
<tr>
<td>Control</td>
<td>2.3</td>
<td>17</td>
<td>150</td>
</tr>
<tr>
<td>0 mM K, 10 mM NaCl</td>
<td>2.2</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>Control</td>
<td>2.1</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>0 mM K, 25 mM NaCl</td>
<td>2.1</td>
<td>21</td>
<td>32</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.0</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>0 mM K, 50 mM NaCl</td>
<td>2.1</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>Control</td>
<td>2.0</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>0 mM K, 75 mM NaCl</td>
<td>2.2</td>
<td>47</td>
<td>34</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.2</td>
<td>52</td>
<td>33</td>
</tr>
<tr>
<td>0 mM K, 100 mM NaCl</td>
<td>1.9</td>
<td>59</td>
<td>26</td>
</tr>
<tr>
<td>Control</td>
<td>2.1</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td>0 mM K, 125 mM NaCl</td>
<td>2.1</td>
<td>92</td>
<td>43</td>
</tr>
<tr>
<td>Control</td>
<td>1.9</td>
<td>102</td>
<td>47</td>
</tr>
<tr>
<td>0 mM K, 150 mM NaCl</td>
<td>2.2</td>
<td>96</td>
<td>54</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.3</td>
<td>108</td>
<td>45</td>
</tr>
</tbody>
</table>

* Determined from [3H]H2O/[14]Cureos uptake data. The values are the averages of 8 experiments. While there was little change from one condition to another in any given experiment, the average value for cell volume from one experiment to another varied over a range of 1.7-2.5 picoiters/cell.

**Fig. 2.** The influence of insulin on 45Rb+/K+ pumping in adipocytes. Suspensions of cells were incubated as described in Fig. 1. At 20 min of incubation either buffer (open symbols) or 0.15 mM insulin (filled symbols) was added. The data are the average of 4 experiments and are expressed as the per cent of the maximum 45Rb+/K+ pumping for each form of the enzyme (C, O, a(+) A, a(+)). The maximum values were 1.7 and 5.0 nmol/min/3 x 10^6 cells for a and a(+), respectively. A, shows pumping as a function of extracellular [Na+]. B, is the same data as in A transformed using the information in Table II, so that the abscissa is expressed in terms of calculated intracellular [Na+]. The curves represent the best fit of the data to a model where 3 sodium ions are required for activity. The K0.5 values are: panel A, control (a), 22 mM; insulin (a), 15 mM; control (a(+)), 85 mM; insulin (a(+)), 55 mM; panel B, control (a), 17 mM; insulin (a), 14 mM; control (a(+)), 52 mM; insulin (a(+)), 33 mM.

**Fig. 3.** Sodium dependence of (Na+,K+)-ATPase activity in adipocytes. The rate of ouabain-inhibitable (Na+,K+)-ATPase activity in adipocyte membranes (O—O), axolemmal membranes (A—A), and kidney microsomes (□—□) was measured at 30°C using the enzyme-coupled spectrophotometric assay of Cantley and Josephson (1976). The data shown are the average of 2 experiments and are expressed as a per cent of maximal activities measured in the presence of either 10 mM KCl (panel A) or 100 mM KCl (panel B). The maxima for each preparation were very similar under either condition and were 50 nmol/min/mg membrane protein for adipocyte and 2 pmol/min/mg protein for both axolemmal and kidney membranes. The curves represent the best fit of the data to a model with 3 sodium ions required for activity. The K0.5 values are: panel A, adipocyte, 3.2 mM; axolemma, 4.5 mM; kidney, 5.9 mM; panel B, adipocyte, 12 mM; axolemma, 16 mM; kidney, 30 mM.

**DISCUSSION**

The data described above clearly demonstrate that the two forms of the Na+ pump in rat adipocytes have quite different affinities for sodium ion in the whole cell. While a has a K0.5 in the range reported for purified enzyme (Robinson and
Flahsner, 1979; Cantley, 1981), the value for \( a(+) \) is considerably higher. Additionally they demonstrate that when fully activated with respect to sodium ion, the amount of \( {^{38}}\text{Rb}/K \) pumping in the whole cell approaches the maximum predicted from \((\text{Na}^+K^-)\text{-ATPase} \) activity of membrane preparations (see Table I). Under these conditions the pumping activity attributable to \( a(+) \) is about three times that of \( a \). This ratio is the same for both front-door and back-door phosphorylation sites and supports the contention that in adipocytes there are three times as many \( a(+) \) molecules as there are \( a \) ones, and that both forms of the enzyme have the same turnover number (Lytton et al., 1985). Measurements of \((\text{Na}^+K^-)\text{-ATPase} \) activity in adipocyte membranes further support these arguments; ouabain inhibition of this activity at 5 \( \mu \)M and 1 mM reveals a ratio of \( a(+) \) to \( a \) activity in the range of 2–3 (data not shown). This would suggest that the only factor limiting \( N^+ \) pump activity in the whole cell is sodium ion affinity.

Indeed, the measured \( K_{0.5} \) values are alone sufficient to explain why the \( N^+ \) pump in whole cells operates at such an unusually low fraction of its total possible activity. Measurements of the resting level of \( N^+ \) within the adipocyte reveal a level of about 15–20 mM (see Table II and Resh et al., 1980). Assuming a model where there are three cooperative \( N^+ \)-binding sites, all of which must be filled for activity (see “Experimental Procedures" for a discussion of this point) and that intracellular \([N^+]\) = 20 mM, then from the \( K_{0.5} \) values for \( a \) of 17 mM (control) and 14 mM (insulin) one can calculate fractional activities of 62 and 74%, respectively, compared to the measured value of 45% under either condition (Lytton et al., 1985). Although the predicted and actual numbers do not match well, one should note that these values fall near the inflection point of the activity curve for \( a \); hence a small error in determining \( K_{0.5} \) or intracellular \([N^+]\) would lead a relatively large change in fractional activity. For \( a(+) \) the \( K_{0.5} \) values are 52 and 33 mM in the presence and absence of insulin; this corresponds to fractional activities of 5 and 18%, compared to the measured values of 5 and 23%, respectively.

It appears that the data fit into a cohesive model where the adipocyte has three times as many copies of the “\( N^+ \)-insensitive” form of the \( N^+ \) pump (\( a(+) \)) as of the “housekeeping" enzyme (\( a \)). However, under basal conditions the activity of \( a(+) \) is suppressed due to the normal sodium level within the cell. Only large increases in sodium (in excess of 25 mM) or regulation of its affinity with the sodium level remaining unchanged could produce a significant stimulation of \( a(+) \). Insulin appears to mediate its effect through the latter mechanism. What remains obscure is just how this is accomplished.

The \( N^+ \) affinity of \((\text{Na}^+K^-)\text{-ATPase} \) in adipocyte membranes is a feature which is possibly relevant to the mechanism of insulin’s action on the \( N^+ \) pump. Despite the large difference between the \( N^+ \) affinity of \( a \) and \( a(+) \) in the whole cell, once the membranes have been isolated, there is no apparent low affinity component, and the measured \( N^+ \) \( K_{0.5} \) matches that reported in the literature for brain (Urayama and Nakao, 1979; Robinson, 1977), which is also rich in \( a(+) \) (Sweedner, 1979). It is, therefore, not surprising that one should be unable to measure an effect of insulin on the \( N^+ \) pump in membranes. It is not clear why Gavryck et al. (1975) do see an insulin stimulation of \((\text{Na}^+K^-)\text{-ATPase} \) in frog muscle membranes. However, the very large amount of insulin used (\( \sim 3 \mu \)M), storage of the membranes at \(-6^\circ \)C for 6–9 days, as well as the source of the membranes (frog muscle versus rat adipocyte) are major differences in experimental protocol. These may account for the opposite results.

It is possible that the same mechanism which insulin em-
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Sweadner, K. J. (1979) J. Biol. Chem. 254, 6060–6067

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