Insulin Action in Isolated Rat Thymocytes

I. BINDING OF ¹²⁵I-INSULIN AND STIMULATION OF α-AMINOISOBUTYRIC ACID TRANSPORT

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SUMMARY

Rat thymocytes, isolated from 13- to 19-day-old animals, responded to insulin with an increased influx of α-aminoisobutyric acid (AIB). Insulin was effective at 1 nM; the half-maximal response was 40 nM, and a maximal displacement was seen at 1 μM. Biologically inactive insulin derivatives as well as adrenocorticotropic hormone and glucagon were without effect. ¹²⁵I-Insulin bound specifically to rat thymocyte receptors and was displaced by unlabeled insulin at 1 nM. The insulin concentration that produced half-maximal displacement of ¹²⁵I-insulin was 20 nM, and complete displacement was seen at 1 μM. Other hormones or inactive insulin derivatives had trivial effects.

Insulin increased AIB influx by both raising the maximal influx capacity (Jₘmax) and lowering the concentration of AIB at which influx was half maximal (Kₘ). Insulin did not affect AIB fractional efflux. The full effect of insulin on influx was not immediate; rather, stimulation of influx increased linearly with time. Cycloheximide both inhibited basal AIB influx and reduced the response to insulin. Insulin had only a small effect on [¹⁴C]leucine incorporation into cellular proteins and had no effect on the uptake of 3-O-methylglucose.

The data suggest that (d) insulin stimulation of AIB transport on rat thymocytes correlates in sensitivity and specificity with ¹²⁵I-insulin binding to receptors, and (b) insulin stimulation of AIB influx may be mediated by changes in synthesis or degradation of the AIB transport apparatus.

Recently it has been demonstrated that human circulating lymphocytes have membrane insulin receptors which are very similar in affinity and specificity to those seen in liver and fat (1-3). The full significance of this finding is not clear because studies of lymphoid tissue have not agreed on a cellular process which insulin regulates (4-6). In the study of hormone-cell interactions lymphoid tissue has certain advantages not shared by other insulin-sensitive tissues. Large numbers of lymphocytes are readily obtained in normal and pathological states, and homogeneous cell suspensions of this tissue can be prepared without the use of digestive enzymes. Thus it is important to determine whether the insulin receptors on the cell surfaces of lymphocytes are linked to the regulation of a cell function.

Because insulin stimulates the transport of amino acids in a variety of tissues (7-12) and this effect is easily quantitated in cell suspensions, we undertook the study of insulin effects on the transport of α-aminoisobutyric acid in lymphoid tissue (13-15). The studies reported here describe the transport of AIB in suckling rat thymocytes, the effect of insulin on this process, and the correlation between insulin receptor binding and insulin stimulation of transport.

EXPERIMENTAL PROCEDURE

Materials--Suckling Sprague-Dawley rats, 11 days old, were purchased from Taconic Farms, Inc.; [¹⁴C]AIB (5.3 mCi per mmole), 3-O-methyl[¹⁴C]glucose (18.5 mCi per mmole), [¹⁴C]-leucine (327 mCi per mmole), and [¹³¹I]mannitol (3.14 mCi per mmole) from Nuclear-Chicago; cycloheximide and adrenocorticotropic hormone from Sigma; carboxymethylated A and B chains of insulin from Schwartz/Mann; AIB and ouabain from Calbiochem; bovine serum albumin from Armour; and nylon mesh screen (#114) from Nyrex. Scintillation fluid used in these studies consisted of 15 parts of toluene (J. T. Baker Chemical Co.), 5 parts of Triton X-100 (New England Nuclear), and 1 part of Liquifluor (New England Nuclear).

The following were provided as gifts: porcine insulin (25 units per mg), porcine proinsulin (015-984B-99-C), and glucagon from Lilly, desalamin-deasparagine bovine insulin (JG-11-21-10) from Dr. H. Carpenter, and biologically active ¹²⁵I-insulin (116 mCi per mg) from Dr. R. Kahn (1, 2).

Other chemicals were of the highest grade of purity obtainable from commercial sources.

Preparation of Isolated Thymocytes--Rats of both sexes 13 to 19 days old were anesthetized with ether. To minimize contamination with red cells the abdominal vessels were cut and the animal exsanguinated. The thymus was exposed, gently removed in small pieces with a 5-inch dental forceps, and placed in 5 ml of iced buffer containing 120 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 2.5 mM MgCl₂, 1.5 mM NaH₂PO₄, 22 mM glucose, 20 mM HEPES, 100 mM NaF, 10 mM sodium pyruvate, 10 mM sodium pyruvate, and 100 units per ml heparin. The thymus was rinsed in 5 ml of iced buffer containing 120 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 2.5 mM MgCl₂, 1.5 mM NaH₂PO₄, 22 mM glucose, 20 mM HEPES, 100 mM NaF, 10 mM sodium pyruvate, 10 mM sodium pyruvate, and 100 units per ml heparin.

The abbreviation used is: AIB, α-aminoisobutyric acid.
mg per ml of bovine serum albumin and 25 mM Tris-HCl (pH 7.4) 2.

The glands were carefully teased in a plastic Petri dish with two 5-inch dental forceps until the buffer became cloudy; fragments were allowed to settle, and the supernatant was aspirated with a Pasteur pipette into an iced beaker. Three milliliters of iced buffer were then placed into the Petri dish and the glands again teased until no additional cells could be removed from thymic strona. The two suspensions were combined, filtered through nylon screen, and then centrifuged at 100 × g max at 4° for 10 min. The supernatant was decanted and 2 ml of the above buffer with the glucose reduced to 11 mM were added to the sediment and the cells suspended by gentle agitation.

Thymocytes prepared in this manner were 95% viable as measured by the exclusion of 0.1% trypan blue and remained so for up to 4 hours in the presence and absence of insulin. Both control and insulin-treated cells showed identical uptake of [3H]AIB when gassed with either 100% O2 or ambient air.

**AIB Uptake—**Uptake was measured by a modification of the method of Gardner and Levy (16) as described for human erythrocytes. Cells (20 to 70 × 10^6 per ml) were incubated with gentle shaking at 37° in 25-ml Erlenmeyer flasks with 3 ml of the above-mentioned buffer containing 11 mM glucose (incubation medium). At appropriate times, after addition of [3H]AIB, 100-μl samples were added to 250 μl of iced buffer in a micro test tube and centrifuged for 15 s at 10,000 × g in a Beckman 152 microfuge. The supernatant fluid was then aspirated and the cell pellet washed and centrifuged twice more with 250 μl of iced buffer. To release [3H]AIB from the washed cell pellet, 100 μl of 10% perchloric acid were added to the washed cell pellet and the mixture agitated. The tube was centrifuged and the tube and its contents inverted and placed into a counting vial containing 20 ml of scintillation mixture; the vial was agitated until the contents of the tube were dispersed. To determine the [3H]AIB concentration in the incubation mixture, 100 μl were added to 100 μl of 10% perchloric acid in a micro test tube and the mixture agitated, centrifuged for 45 s, and dispersed into 20 ml of scintillation fluid. [3H]AIB taken up by the cells was calculated by obtaining the ratio of intracellular [3H]AIB to total [3H]AIB in the incubation mixture. Variations in quenching were negligible when evaluated by an automatic external standard. Using [14C]amino acid as an external standard, less than 0.1% of the [14C]AIB radioactivity remained with the cell pellet after the washing procedure.

The radioactivity taken up by thymocytes consisted entirely of [3H]AIB as determined with a Beckman model 120B amino acid analyzer in series with a flow cell in a Packard Tri-Carb liquid scintillation spectrometer. Other studies revealed that less than 0.26% of the [3H]AIB radioactivity taken up by the cells was incorporated into protein as determined by [14C]incorporation into perchloric acid-precipitable material.

**125I-Insulin Binding—**125I-Insulin binding to rat thymocytes was performed by the method of Gavin et al. (1) as described for human lymphocytes. Cells were incubated with 125I-insulin at 15° in 0.5 ml of buffer containing 112 mM NaCl, 5 mM KCl, 2.5 mM MgSO4, 10 mM glucose, 1.2 mM EDTA, 22 mM sodium acetate, 20 mg per ml of bovine albumin, and 50 mM Tris-HCl, pH 7.4. Binding was maximum after 1 hour and remained constant over the next hour. At the end of the incubation, duplicate 200-μl aliquots were added to 150 μl of buffer in a micro test tube and centrifuged at 10,000 × g for 1 min in a Beckman 152 microfuge. The supernatants were aspirated and the tips of the tubes which contained the cell pellets were counted in a crystal scintillation counter.

**Insulin Levels in Rats—**Insulinoassayable insulin was determined in plasma from four 13-day-old rats using a solid phase radioimmunoassay (17). Insulin levels ranged from 8 to 16 microunits per ml (10 ± 9 microunits per ml, mean ± S.D.; about 70 pm).

**RESULTS**

**AIB Uptake; Effect of Insulin—**Insulin at 3 μM stimulated uptake of [3H]AIB into rat thymocytes (Fig. 1). After 30 min of incubation insulin increased uptake by only 12%, but the insulin effect gradually increased to a maximum of 33% at 2 hours when a steady state was reached. Rat thymocytes were able to accumulate AIB against a concentration difference. Assuming that thymic lymphocytes are spheres 8 μm in diameter and contain 78% water (4, 13), the steady state ratio of intracellular AIB to extracellular AIB was 16 and 12 in the presence and absence of insulin, respectively.

**AIB Influx; Effect of Insulin—**The uptake of AIB was the net result of influx (velocity of AIB entry per unit area) and efflux (velocity of AIB exit per unit area). Because of transconcentration effects (the ability of intracellular amino acids to influence the influx of extracellular amino acids) it was not possible to derive values for the kinetics of influx from the uptake equations derived for AIB transport in muscle (9). In thymocytes, when the intracellular concentration of AIB was increased by previously incubating cells for 2 hours in the presence of various concentrations of AIB, there was a progressive decrease in AIB influx as the preliminary incubation concentration of AIB was increased (Fig. 2). Transconcentration effects were seen in cells previously incubated in with as little as 10 μM AIB; with 1 μM AIB, AIB uptake was reduced by 50%.

AIB influx was measured by observing the initial velocity of AIB uptake. After 2 hours of preliminary incubation, in both the presence and absence of insulin, AIB uptake as a function of time was linear for at least 16 min after the addition of [3H]AIB (Fig. 3). Therefore, in all subsequent studies, AIB influx was calculated by obtaining the ratio of intracellular [3H]AIB to total [3H]AIB in the incubation mixture, 100 AIB, AIB uptake was reduced by 50%.

The effects of previously incubating cells with or without insulin were studied. In the absence of insulin, AIB influx increased over the first hour and then remained constant during the subsequent 2 hours (Fig. 4). This rise in control AIB influx presumably reflects adaptation of the thymocytes to the incubation medium. In contrast, in the presence of insulin, AIB influx increased progressively throughout the 3-hour incubation period. When the effect of insulin on AIB influx was calculated by subtracting control influx values from those obtained in the presence of insulin, the effect of insulin increased linearly with time over the 3-hour incubation period.

AIB influx was dependent on the extracellular AIB concentration (Fig. 5). Below 5 mM there was a rapid increase in influx with increasing AIB extracellular concentrations. At concentrations above 5 mM there was an apparent linear relation between AIB influx and extracellular AIB concentration. This biphasic dependence of influx on the extracellular AIB concentration can be resolved into two components, a hyperbolic component (saturable) and a linear component (un saturable) (9, 13). The linear component of AIB influx (Fig. 5) was determined by computing the product of the slope and the ex-
MINUTES OF INCUBATION

FIG. 1 (left). Effect of insulin on AIB uptake. In this experiment 42 × 10⁶ cells per ml were incubated with 30 μM [¹⁴C]AIB in the presence and absence of 3 μM insulin for up to 3 hours. AIB is plotted as a function of duration of incubation. Each point is the mean of triplicate determinations ± the standard deviation.

Fig. 2 (center). Effect of preliminary incubation with AIB on AIB influx. In this experiment 23 × 10⁶ cells per ml were incubated in the presence of several concentrations of AIB for 2 hours. The cells were then added to 10 ml of iced incubation medium and centrifuged at 200 × g for 5 min at 4°C. The cells were washed twice in 12 ml of iced incubation medium and resuspended in incubation medium warmed to 37°C. [¹⁴C]AIB was then added to give a concentration of 30 μM, and uptake was determined at 6 and 12 min. Each point is the mean of duplicate determinations.

FIG. 3 (right). Effect of insulin on the AIB influx. In this experiment 40 × 10⁶ cells per ml were previously incubated for 2 hours in the presence and absence of 3 μM insulin; [¹⁴C]AIB was then added to give a final concentration of 30 μM. AIB uptake is plotted as a function of duration of incubation. Each point is the mean of duplicate determinations.

tracellular AIB concentration at each AIB concentration studied. These values were then subtracted from the measured influx values to give values for the hyperbolic component (Fig. 5). Thus, measured AIB influx (Jₘ) could be related to extracellular AIB concentration by the equation

\[ J_{in} = \frac{J_{max}[A]}{K_a + [A]} + S_{lin}[A] \]

where \( J_{max} \) was the maximal AIB influx which was described by

Fig. 4. The effect of preliminary insulin incubation on AIB influx. In this experiment 3 ml of 20 × 10⁶ cells per ml were added to 20 flasks, and insulin was added to alternate flasks to give a concentration of 3 μM. At the times indicated [¹⁴C]AIB was added to a control and insulin flask, respectively, to give a concentration of 30 μM; AIB influx was determined after 5 min. Each point is the mean of duplicate determinations.

Fig. 5. AIB influx as a function of extracellular AIB concentration. 42 × 10⁶ cells per ml were incubated for 2½ hours. [¹⁴C]AIB was then added to give concentrations of 0.2 to 30 mM, and AIB influx was determined after 10 min. Measured influx curve (○) has been resolved into two components; a linear component (□) and a hyperbolic component (●) (see text). Each point is the mean of duplicate determinations.
FIG. 6. The effect of insulin on AIB measured influx. In this experiment \(3 \times 10^6\) cells per ml were incubated in the presence and absence of 3 \(\mu\)M insulin for 2\(\frac{1}{2}\) hours. [\(^{14}\)C]AIB was then added to give concentrations of 0.2 to 20 mM, and AIB influx was determined after 10 min. Each point is the mean of duplicate determinations. Inset, effect of insulin on the linear portion of the AIB measured influx curve. In this experiment 26 \(\times 10^6\) cells per ml were incubated in the presence and absence of 3 \(\mu\)M insulin for 2\(\frac{1}{2}\) hours. [\(^{14}\)C]AIB was then added to give concentrations of 5.5 to 20 mM and AIB influx was determined after 10 min. The control slope was 0.12 \(\pm\) 0.006 pmoles per 10\(^9\) cells per hour per mM AIB (mean \(\pm\) S.D.) and the control intercept was 3.74 \(\pm\) 0.09 pmoles per 10\(^9\) cells per hour. The insulin slope was 0.24 \(\pm\) 0.019 and the insulin intercept was 5.15 \(\pm\) 0.28 pmoles per 10\(^9\) cells per hour. Each point is the mean of duplicate determinations.

The hyperbolic component, \(K_m\), was the AIB concentration at which the influx of the hyperbolic component was half-maximal, and \(S_c\) was the slope of the linear component. When \(A \gg K_m\), the equation reduced to

\[ J_{in} = J_{max} + S_c[A] \]

illustrating that the value of \(J_{max}\) could be determined by extrapolating the line describing AIB influx measured at relatively high AIB concentrations to the intercept on the y-axis (Fig. 5).

Using this analysis, insulin had three effects on AIB influx which were demonstrated in the experiments seen in Figs. 6 and 7. First, insulin raised the \(J_{max}\) of the hyperbolic component from 4.4 \(\pm\) 0.3 to 5.5 \(\pm\) 0.4 pmoles per 10\(^9\) cells per hour (mean \(\pm\) S.D. \(P < 0.01\)) (Figs. 6 and 7). Second, insulin also lowered the \(K_m\) of the hyperbolic component from 0.77 \(\pm\) 0.03 mM to 0.53 \(\pm\) 0.03 mM (mean \(\pm\) S.D. \(P < 0.01\)) (Fig. 7). In two other experiments insulin lowered the \(K_m\) from 1.15 \(\pm\) 0.01 mM to 0.63 \(\pm\) 0.025 mM and from 0.76 \(\pm\) 0.04 mM to 0.50 \(\pm\) 0.06 mM. Finally, insulin increased the slope of the linear component from 0.12 \(\pm\) 0.006 to 0.19 \(\pm\) 0.005 pmoles per 10\(^9\) cells per hour per mM AIB (\(P < 0.001\)) (Fig. 6).

**Efflux Studies; Effect of Insulin**—Insulin did not alter AIB fractional efflux (Fig. 8). The natural logarithm of the fraction of the intracellular AIB originally present as a function of time was linear for at least 30 min and was not changed by insulin. Total AIB efflux in cells incubated with insulin (Table I) was greater than in cells incubated without insulin; however, this was an indirect effect attributable to the effect of insulin on AIB influx which raised the intracellular AIB concentration and thereby increased AIB efflux.

**Other Effects on Influx**—Replacing the sodium in the incubation medium with choline significantly reduced AIB influx;
TABLE I

Effect of insulin on AIB influx

In this experiment 44 × 10^6 cells were previously incubated for 2 hours with several concentrations of [14C]AIB in the absence and presence of 3 μM insulin. Efflux was then determined over the next 30 min, as described in Fig. 8.

| Additions to preliminary incubation medium | Intracellular AIB concentration after preliminary incubation | Fractional efflux coefficient (min⁻¹) | AIB influx
|-------------------------------------------|------------------------------------------------------------|-------------------------------------|----------------
|                                           | nmoles/10^9 cells | mean ± S.D. | nmoles/10^9 cells/min |
| AIB (30 μM)                                | 126              | 0.0248 ± 0.002 | 3.1                |
| AIB (30 μM) + insulin                      | 161              | 0.0245 ± 0.002 | 4.0                |
| AIB (90 μM)                                | 227              | 0.0251 ± 0.007 | 5.7                |

TABLE II

Effects of Na⁺ and ouabain on AIB influx

In this experiment 24 × 10^6 cells per ml were incubated for 2 hours in the presence and absence of insulin (3 μM) in media containing either 120 mM choline chloride or NaCl. Ouabain was then added to appropriate tubes; 5 min later [14C]AIB was added to give a concentration of 30 μM, and influx was determined after 10 min. Each point is the mean of duplicate determinations.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>AIB influx</th>
<th>(Insulin minus control/Control) X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/10^9 cells/hr</td>
<td></td>
</tr>
<tr>
<td>NaCl (120)</td>
<td>184</td>
<td>350</td>
</tr>
<tr>
<td>NaCl (120) + ouabain (0.1)</td>
<td>153</td>
<td>306</td>
</tr>
<tr>
<td>Choline chloride (120)</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>Choline chloride (120) + ouabain (0.1)</td>
<td>23</td>
<td>41</td>
</tr>
</tbody>
</table>

However, insulin still increased AIB influx in cells which were incubated in the sodium-free medium (Table II). Addition of ouabain, which inhibits insulin stimulation of AIB transport in fetal rat calvaria (19), did not significantly alter AIB influx in cells which were previously incubated with or without insulin (Table II).

Lowering the incubation temperature from 37° to 24° reduced control AIB influx by 75% and lowered the insulin to control AIB influx ratio by 40%. Eliminating glucose from the incubation medium caused a 30% fall in control AIB influx but did not influence the insulin to control AIB influx ratio. Preloading cells with AIB decreased AIB influx. However, this trans-concentration effect did not affect the action of insulin as previously incubating cells with 30 μM or 300 μM AIB reduced control and insulin-stimulated influx by the same proportion (Table III).

Protein Synthesis Studies—Studies in rat diaphragm and fetal rat calvaria have been interpreted as indicating that the effect of insulin on AIB transport is mediated by increased protein synthesis (12, 18, 19). In thymocytes incubation with 0.1 μM cycloheximide, an inhibitor of protein synthesis, reduced both control and insulin-stimulated AIB influx (Fig. 9). At 0.1 μM cycloheximide, 3 the insulin to control ratio approached unity, suggesting that the insulin effect in rat thymocytes may have been mediated in part through increased protein synthesis or decreased protein degradation (20–22). After 30 min of incubation with insulin, [14C]leucine incorporation into perchloric acid-precipitable material was not increased (Table IV) and after 2 hours was only increased by 20%. Insulin also had no effect on uptake of the nonmetabolizable glucose analogue 3-O-methylglucose (Table V).

Stimulation of AIB Influx as a Function of Insulin Concentration—An effect of insulin on AIB influx could be detected at insulin concentrations as low as 5 ng per ml (0.9 nM) and the half-maximal effect of insulin occurred at approximately 40 nM (Fig. 10). Maximal effects were seen at 1 μM. The insulin analogue, desalannine-desaspartagine insulin, which has 1 to 2% of the activity of native insulin (1, 2) as well as insulin A chain, insulin B chain, glueagon, and adrenocorticotropie hormone, did not alter AIB influx.

Binding of 125I-Insulin—Insulin stimulation of AIB influx correlated with 125I-insulin binding to receptors in rat thymocytes. Because large numbers of cells were necessary to meas-
**Table IV**

_Effect of insulin on [14C]leucine incorporation into protein_

In this experiment 80 x 10^4 cells per ml were incubated with 2 µM [14C]leucine in the presence and absence of 3 µM insulin. At appropriate times 200 µl of the cell suspension were mixed with 12 ml of fixed buffer which contained 140 mM NaCl, 5 mM KCl, and 10 mM NaHPO_4/NaHPO_4, pH 7.4. Cells were then centrifuged at 500 X g for 5 min, the supernatant decanted, and the cell pellet treated with 12 ml of 10% perchloric acid. After addition of 0.4 mg of bovine albumin the mixture was centrifuged at 500 X g for 5 min, the supernatant decanted, the pellet solubilized in scintillation fluid, and the radioactivity counted. Results are the means ± S.D. for triplicate determinations.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>30 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>1.07 ± 0.01</td>
<td>3.34 ± 0.19</td>
</tr>
<tr>
<td>Absent</td>
<td>1.08 ± 0.05</td>
<td>2.80 ± 0.16</td>
</tr>
</tbody>
</table>

**Table V**

_Effect of insulin on 3-O-methylglucose uptake_

In this experiment 70 x 10^4 cells per cc were incubated for 1 hour in the presence and absence of 3 µM insulin. 3-O-Methyl-[14C]glucose was then added to give a concentration of 35 µM, and uptake was determined after 1 hour. Results are the means ± S.D. for triplicate determinations.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>3-O-Methylglucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>2.64 ± 0.17</td>
</tr>
<tr>
<td>Absent</td>
<td>2.43 ± 0.09</td>
</tr>
</tbody>
</table>

Discussion

We demonstrated in suckling rat thymocytes that insulin stimulates amino acid transport. The effect of insulin (7-9) and other anabolic hormones (24) to stimulate amino acid uptake is a well studied physiological phenomenon. Recently, the interaction of insulin with specific membrane receptors has been quantitated in several tissues (1-3, 25). In this present study with rat thymocytes we have related stimulation of AIB uptake by insulin to its occupancy of specific membrane receptors.

There are certain similarities between AIB transport in suckling rat thymocytes and guinea pig lymph node cells (13). In both tissues measured influx can be divided into two components, one hyperbolic and one linear. Furthermore, both types of lymphocytes can transport AIB against a concentration difference. However, the K_m of the hyperbolic transport component is 7-fold lower in guinea pig lymphocytes (0.11 mM) than in rat thymocytes. In contrast, human circulating lymphocytes were reported to have only one component of measured AIB influx which was hyperbolic and had a K_m of 2.0 mM (15). In neither guinea pig lymph node cells nor human circulating lymphocytes was an effect of insulin reported.

Lypolysis and glucose oxidation in isolated rat fat cells are sensitive to insulin (9, 3, 26, 27) and this tissue has an AIB transport system with a K_m of 0.7 mM (26) which is similar to the one in rat thymocytes. However, in fat cells under basal conditions insulin does not increase AIB transport (26, 27) but does accelerate the incorporation of [14C]leucine into protein (26).

In rat thymocytes, insulin altered both the K_m and J_max of the hyperbolic component of AIB influx. It was observed that in chicken embryo heart (10), fetal rat calvaria (12), and diaphragms of 10-day-old rats (19), insulin acted to change J_max of this component, whereas in 25-day and older rats insulin acted to change only the K_m (9, 19). Thus, changes in J_max may be a characteristic of fetal and newborn animals, whereas changes in K_m may be a characteristic of older animals. Since our animals were intermediate in age, the finding of changes in both parameters is consistent with these observations.

Although the mechanism(s) by which insulin influences amino acid transport are unknown, extracellular sodium is believed to...
been shown to stimulate protein synthesis or decrease protein across the plasma membrane; it did not increase the uptake of
Thus, sodium may not be essential for the action of insulin in
The full effect of insulin was not immediate; rather, AIB influx and other hormones. In this experiment 200 × 10^6 cells per ml
were incubated at 15° for 80 min with a tracer amount of ^3H-insulin (0.12 nM) in the absence and presence of unlabeled insulin or other hormones. The 0% binding, which represents the percentage of the ^3H-insulin radioactivity bound in the presence of an excess of unlabeled insulin (100 μg per ml) was 1.4 ± 0.10 (mean ± S.D.) of the total radioactivity. The 100% binding, which represents ^3H-insulin bound in the absence of unlabeled insulin was 4.18 ± 2.6. Each point is the mean of quadruplicate determinations. ACTH, adrenocorticotropic hormone; Desal-Desasp, desalanine-desasparagine insulin.

he involved. The presence of sodium is required for active (uphill) transport of some amino acids and sugars (28), and in studies of AIB transport in rat diaphragm and fetal rat calvaria (12, 28) replacement of sodium by choline both reduced uphill transport and abolished the insulin effect. However, we found that in rat thymocytes, replacement of sodium with choline markedly reduced basal influx but the effect of insulin persisted. Thus, sodium may not be essential for the action of insulin in thymocytes.

It has been proposed (12, 18) that amino acid transport in mammalian cells is mediated by a membrane carrier protein that has a short half-life (29). We found in rat thymocytes that cycloheximide, an inhibitor of protein synthesis, inhibited insulin stimulation of AIB uptake in chicken embryo hearts (10) and fetal rat calvaria (12), rat diaphragm and levator ani muscle being 10-fold more sensitive (9, 31). However, in all of these tissues including thymocytes the concentration of insulin required to produce maximum stimulation of AIB uptake is in excess of physiological levels.

Insulin binding to receptors in isolated cells has been studied directly in fat cell suspensions prepared from adipose tissue with the use of the digestive enzyme collagenase (3, 25). In adipose tissue insulin stimulates glucose oxidation half-maximally at about 50 pm and maximally at about 200 pm (2, 3, 25, 26). However, attempts to correlate insulin-receptor binding and this biological function have revealed complicated relations. In one study (8) a dissociation constant of 61 pm was calculated, whereas in another one of 8 nm was found (25). Further, both studies revealed that the dose-response curve for insulin binding was much broader than that for glucose oxidation, as maximum displacement of ^3H-insulin binding occurred at unlabeled insulin concentrations of 100 nm.

We measured directly the binding of insulin to receptors in rat thymocytes and found that the displacement of ^3H-insulin correlated with insulin stimulation of AIB influx. With both, an effect of insulin was seen at 5 ng per ml (0.9 nM), and maximal effects were seen at 5 μg per ml (0.9 μM). The relatively broad AIB and dose-response curve for insulin was due to two binding sites for insulin which had dissociation constants of 9 nm and 100 nm.

Further, we found that only insulin both displaced ^3H-insulin from its receptors and stimulated AIB influx. Biologically inactive or partially active insulins as well as other hormones had little or no effect on either process. Therefore, the data demonstrated that stimulation of AIB influx by insulin was directly proportional to the number of receptor sites occupied by insulin. Since these insulin receptor studies in rat thymocytes reflect insulin binding which is directly related to regulation of a cellular process, we believe that such studies may be an important tool to further investigate the action of insulin.

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