Adenosine Triphosphate-dependent Inhibition of Insulin-stimulated Glucose Transport in Fat Cells

POSSIBLE ROLE OF MEMBRANE PHOSPHORYLATION*

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SUMMARY

Addition of low concentrations of ATP, but not other nucleoside triphosphates, to fat cells inhibits insulin-stimulated glucose oxidation under conditions that measure glucose transport. Basal rates of glucose transport are not inhibited. The inhibition cannot be overcome by increasing the concentration of insulin, and the dose-response relationships indicate no gross alteration in the apparent affinity of the cells for the hormone. The binding of 125I-labeled insulin is not altered by ATP. Under the conditions used, ATP does not alter oxidation by affecting cytoplasmic metabolic processes. The effects of exogenous ATP on glucose transport were confirmed by direct measurements of 3H-labeled 3-O-methyl-D-glucose uptake in isolated fat cells. ATP inhibits the insulin-stimulated but not the basal rate of 3-O-methylglucose transport.

The inhibition by ATP is relatively selective since it represents a fall in the apparent affinity for glucose of the insulin-activated component of the glucose transport system. The inhibition can be overcome by high glucose concentrations. The antilipolytic activity of insulin in ATP-treated cells is unaffected while insulin-stimulated transport is markedly depressed; these activities of insulin can thus be dissociated. ATP also inhibits the enhanced rates of glucose transport observed with concanavalin A, wheat germ agglutinin, spermine, spermidine, cysteine, and glutathione, substances that activate the same glucose transport system as insulin. The ouabain-stimulated glucose oxidation, which occurs by mechanisms different from those of insulin, is not affected by ATP.

The ATP analog, α,β-methylene-adenosine 5'-triphosphate (Ap(CH2)pp), can mimic the effect of ATP. Another analog, β,γ-methylene-adenosine 5'-triphosphate (App(CH2)p), does not suppress insulin-stimulated transport but it can effect selectively inhibit the ATP effect; this phosphonic acid analog cannot donate its terminal phosphoryl moiety in phosphorylation reactions. Phloretin, a strong competitive inhibitor of D-glucose transport, can protect the cells from the ATP effect. Phlorizin, another competitive inhibitor, potentiates the effect of ATP.

Fat cells exposed to low concentrations (5 to 50 μM) of ATP for a short time (5 min) at 24°C continue to exhibit a diminished insulin response (glucose transport) after thorough washing to remove the ATP in the medium. The recovery of insulin sensitivity during subsequent incubation at 37°C is slow, pointing to the protracted and relatively irreversible effect of such mild treatment with ATP. Brief treatment of cells with [γ-32P]ATP results in the selective phosphorylation of two plasma membrane components which are estimated to be of molecular weight 16,000 and 22,000 by sodium dodecyl sulfate gel electrophoresis. Phosphorylation of the latter is inhibited by phloretin, and phosphorylation of both is inhibited by App(CH2)p.

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The possibility is entertained that the procedures utilized may selectively label membrane components involved in insulin-mediated glucose transport (glucose carriers). Consideration is given to the possible physiological significance of such selective membrane phosphorylation in the control of insulin sensitivity in normal as well as insulin-resistant states.

In the last few years considerable progress has been made toward understanding the nature of insulin-receptor interactions in intact fat cells and in membrane preparations derived from these cells (1). Furthermore, it is now known that insulin can under certain circumstances decrease the intracellular levels of cyclic AMP (2–6) and increase the levels of cyclic GMP (7), and

1 The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; cyclic GMP, cyclic guanosine 3':5'-monophosphate; Ap(CH2)pp, α,β-methylene-adenosine 5'-triphosphate; Ap(CH2)p, α,β-methylene-adenosine 5'-diphosphate; App(CH2)p, β,γ-methylene-adenosine 5'-triphosphate.
that the hormone can directly decrease the basal or stimulated activity of membrane bound adenylate cyclase in subcellular systems (8-12). Despite these advances, it is not known whether these or other processes are involved in the mechanism by which insulin modifies the fundamental processes involved in sugar translocation across mammalian cell membranes.

Adipose tissue cells (13-26) and plasma membrane vesicle preparations (27-30) derived from these cells have proven very useful as models for elucidating the basic kinetic properties of glucose transport and its modification by insulin. The accumulated data support the notion that in adipose tissue, glucose transport occurs by a carrier-mediated (probably "mobile"), facilitated diffusion mechanism which exhibits bidirectionality, stereospecificity, temperature sensitivity, saturability, independence from energy-producing processes, inhibition by phloretin, and insensitivity to ouabain (14, 16, 23, 30). Insulin enhances transport in either direction principally by increasing the apparent affinity of the carrier system for glucose (14, 16, 27). Provided low concentrations of glucose are utilized, measurements of the conversion of 4C-labeled glucose to 14CO2 in intact cells reflect glucose transport in the absence or presence of insulin since under these conditions the rate of glucose entry into cells is the rate-limiting step in glucose oxidation and metabolism (13, 14, 16, 18).

Despite the availability of suitable simple systems for study, as well as the knowledge which has been gained concerning the kinetic properties of glucose transport, very little is known concerning the structural components involved in glucose transport or the chemical basis for the control of such processes in the intact cell.

It has recently been reported that low concentrations of exogenously added ATP and the phosphonic acid analog of ATP, Ap(CH2)pp, but not App(CH2)p, inhibit insulin-stimulated glucose oxidation in isolated fat cells (31). These results suggested the possibility that hydrolytic cleavage or transfer of the γ-phosphate group of ATP may be involved in a process which specifically modifies glucose transport. The present studies, which describe in detail these effects of ATP, suggest the existence of a highly specific and facile phosphorylation reaction of superficial membrane components which may be directly involved in those functions of the glucose carrier system which are specifically stimulated by insulin and other insulin-like substances. These findings may be of special interest in view of the rapidly growing evidence that protein phosphorylation reactions are of major importance in regulating the activity of a variety of enzymes, especially those whose actions are subject to control by cyclic AMP (32).

**MATERIALS AND METHODS**

Isolated fat cells were prepared from 80- to 140-g Sprague-Dawley rats according to the method of Rodbell (15), and glucose oxidation was measured by conversion of D-[U-14C]glucose to 14CO2; 0.2 mM glucose was used as previously described (15, 18, 33, 34). Lipolysis was studied (33) by determining the concentration of glycerol by the method of Ryley (35). The procedures for isolation of insulin and for studying the binding of 125I-insulin to fat cells have been described in detail (36, 37).

The uptake of 3-O-methyl-D-glucose into isolated fat cells was measured by the oil flotation separation technique (38) using Beckman microfuge model 152. Uptake was performed by adding 3-O-methyl-D-[methyl-14H]glucose (specific activity, 1.2 mC per mmole), final concentration 20 μM, to intact cells suspended in Krebs-Ringer bicarbonate buffer of pH 7.4, containing 1% (w/v) albumin. The uptake reaction was stopped at 20-s intervals by transferring 0.1 ml of the cell suspension to a microfuge tube (0.5 ml) containing 0.2 ml of silicone fluid followed by centrifugation for 15 s. The cells, which remained on top, were rapidly washed by adding 50 μl of the same ice-cold buffer followed by immediate collection. The microvessels above the silicone fluid were removed by cutting the tube with a razor blade. The cells were dissolved in 1 ml of 10% sodium dodecyl sulfate, and radioactivity was determined at 40% efficiency in the presence of a scintillation fluid consisting of 10 ml of TLA toluene fluorobutyl (Beckman) and 2 ml of Biosolv solubilizer BBS-5 (Beckman). All samples were performed in triplicate.

Purification of subcellular fractions was performed essentially as described by Avruch and Wallach (39). Briefly, isolated fat cells (from about 10 rats) were suspended in 10 ml of 0.25% isotonic sucrose containing Tris-HCl (10 mM) and 1 mM EDTA, pH 7.4. The cells were homogenized with a Polytron PT-10 (Brinkmann) at a speed of 2,500 rpm for 60 s. The total particulate fraction was obtained by centrifugation at 30,000 × g. The pellet, resuspended in 10 ml of the same buffer, was layered on a 27-ml linear sucrose density gradient, 27.67 to 54.17 (w/v), containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The gradients were centrifuged in a Beckman SW 27 rotor at 25,000 rpm at 4°C for 60 min in a Spinco model L-2-65B ultracentrifuge. The upper microsomal membrane zone, middle mitochondrial, and bottom nuclear fractions were aspirated separately and diluted with 6 volumes of 1 mM EDTA-Tris-HCl buffer, pH 7.4. Insulin was added to suspensions of cells centrifuged at 40,000 × g for 30 min, and the pellets were resuspended in 0.2 M sodium phosphate buffer (pH 7.0) for gel electrophoresis.

Phosphorylation reactions were performed by adding 5 μM γ32PATP (specific activity, 3 × 106 cpm per pmole) to intact fat cells suspended in Krebs-Ringer bicarbonate buffer containing 1% (w/v) albumin. The reaction was stopped by diluting with Krebs-Ringer bicarbonate buffer, 1% albumin, and washing three times with the same buffer. The subcellular fractions were then prepared and purified as described above.

γ32P-labeled subcellular fractions prepared by the above procedure were subjected to electrophoresis in 7.5% polyacrylamide disc gels containing 0.1% sodium dodecyl sulfate, at pH 7.0 in 0.1 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) sodium dodecyl sulfate, 3 mM dithiothreitol, and 2% (w/v) sucrose. Electrophoresis was performed with a current of 5.5 mA per tube for 3 hours. Proteins were detected by staining overnight with Coomassie blue (0.05% in 25% isopropyl alcohol) (40). Molecular weights were estimated by comparisons with the electrophoretic mobilities of standard proteins (cytochrome c, ovalbumin, and serum albumin). The gels were sectioned (about 2-mm sections) with a manual slicing device for assay of radioactivity using Bray's scintillation counting solution.

Nucleoside triphosphates, adenine nucleotides, and ouabain were obtained from Sigma. Ap(CH2)pp, App(CH2)p, and Ap(CH2)p were purchased from P-L Biochemicals. Wheat germ agglutinin was isolated and purified by the method of Marchesi (42). Concanavalin A was obtained from Miles. Cytochalasin B was obtained from Imperial Chemical Industries, England. Phloretin and phlorizin were purchased from K & K Laboratory. γ32P ATP (specific activity, 20 to 30 Ci per mmole) was purchased from New England Nuclear. 3-O-Methyl-D-[methyl-14H]glucose (specific activity, 1.2 Ci per mmole) was purchased from International Chemical & Nuclear Corporation.

**RESULTS**

Effects of ATP on Basal and Insulin-stimulated Rates of Glucose Oxidation—Addition of relatively low concentrations of ATP to the medium does not decrease the basal rates of glucose oxidation of isolated fat cells but it greatly reduces the oxidative response of these cells to insulin (Fig. 1). A small stimulatory response of basal oxidation is observed with concentrations of...
ATP CONCENTRATION, 10^(-7) M

**Fig. 1.** Effects of ATP on basal and insulin-stimulated conversion of d-[U-14C]glucose to ^14CO_2 by isolated fat cells. Fat cells (about 10^6 cells) were incubated for 2 hours at 37° in 1.25 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (w/v) albumin and 0.2 mM d-[U-14C]glucose (5 μCi per μmole) in the absence (●) and presence of 10 microunits per ml of insulin (○).

ATP lower than 10^(-5) m. With higher concentrations of ATP (above 0.8 mM), some additional depression of insulin-stimulated oxidation as well as of basal oxidation is observed. This effect appears to be different from that which is observed with the low concentrations of the nucleotide since the latter selectively alter the insulin-mediated response.

Inhibition of the insulin response cannot be overcome by increasing the concentration of insulin in the medium (Fig. 2). Even concentrations of insulin as high as 10 milliunits per ml (not shown in figure) are relatively ineffective in overcoming the inhibition. The concentration of insulin required to achieve a half-maximal response is not grossly altered by ATP, suggesting that ATP does not affect the affinity of the insulin receptor for the hormone.

**Fig. 2.** Effect of ATP (none, ●; 10^(-7) M, ○; 10^(-4) M, △) on the dose-response relationship of insulin-stimulated conversion of d-[U-14C]glucose to ^14CO_2 by isolated fat cells (as in Fig. 1).

**Fig. 3.** Effect of ATP on kinetics of glucose transport. The effects of ATP on insulin-stimulated glucose oxidation (Figs. 1 to 3) were detected under conditions which presumably measure the specific role of ATP on glucose oxidation is strongly supported by the studies which utilize phosphonic acid analogs of ATP and by those which demonstrate the relatively irreversible effect of this nucleotide (to be described shortly).

It is very unlikely that the ATP-dependent inhibition of insulin-stimulated glucose oxidation results from chelation of divalent cations by the nucleotides. EDTA and EGTA at 1 mM concentrations do not affect glucose oxidation or the inhibitory effect observed with ATP. Furthermore, the concentrations of ATP required for inhibition of glucose oxidation are much lower than the concentrations of Ca^{2+} and Mg^{2+} (1.2 mM) in the incubation medium. Increasing the concentration of MgCl_2 to 10 mM does not alter the inhibitory effect of ATP.

**Effects of ATP on Kinetics of Glucose Transport**—The effects of ATP on insulin-stimulated glucose oxidation (Figs. 1 to 3) were detected under conditions which presumably measure
glucose transport since under these conditions this is probably the rate-limiting step in the oxidative sequence of this sugar (13, 14, 16, 18). This, coupled with the knowledge that insulin enhances glucose oxidation by increasing glucose transport, and that exogenous ATP was effective at initial concentrations 2 to 3 orders of magnitude lower than the known intracellular concentrations (about 1 mM) of ATP (43, 44), suggested that the effects of ATP were on transport processes rather than on subsequent metabolic steps. Studies were performed to delineate further the nature of this apparent effect of ATP on insulin-stimulated glucose transport.

Studies of glucose oxidation at varying concentrations of the sugar demonstrate that ATP inhibition of the insulin-stimulated response is most pronounced with low concentrations of glucose (Fig. 4). Whereas at low concentrations of sugar the ATP inhibition is profound, virtually no effect is detectable at high sugar concentrations. The shape of the dose-response curves is nearly identical, but the curve obtained in the presence of ATP is shifted to higher concentrations by a factor of about 5. This suggests that ATP has markedly affected the apparent affinity of the transport system for glucose without altering the maximal oxidative capacities in the presence of insulin. Furthermore, it is clear that the basal rates of oxidation are not altered by ATP over the entire range of glucose concentration (Fig. 4). It is notable that although increasing the glucose concentration raises the basal rates of oxidation to levels as high and higher than those (less than 10^{-4} M) at which ATP markedly inhibits the insulin response, no effect of this nucleotide is discernible at high sugar concentrations.

In Fig. 5 the kinetic data on glucose oxidation are depicted in Eadie-Hofstee plots. From this data it is clear that, as described earlier (14), the principal effect of insulin on glucose transport is to increase the apparent affinity of the transport system for glucose; a 2-fold increase in the maximal rate of glucose oxidation also is detectable. ATP reduces the apparent affinity of glucose for transport to a value that approaches that observed in the absence of insulin, but it does not modify the maximal rates of glucose transport. The data suggest, furthermore, that the inhibition of transport by ATP in the presence of insulin is of a competitive type.

**TABLE I**

<table>
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<tr>
<th>Addition</th>
<th>Specific ^125I-insulin bound*</th>
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<tbody>
<tr>
<td></td>
<td>1.3 x 10^6 cpm (3 x 10^{-10} M)</td>
</tr>
<tr>
<td>None</td>
<td>7800 ± 300</td>
</tr>
<tr>
<td>ATP (10^{-4} M)</td>
<td>7200 ± 200</td>
</tr>
</tbody>
</table>

* Counts per min bound, average value ± S.E. of triplicates.
gests that these plant lectins may activate the insulin-responsive
carriers which are stimulated by insulin. Since these results suggest that
ouabain does not stimulate glucose oxidation by activating the same
processes as insulin, it was of interest to determine the effect of ATP on its stimulatory response.

Exogenous ATP does not inhibit the ouabain-stimulated rate of glucose oxidation (Fig. 7). Even in the presence of insulin, the stimulatory effect of ouabain is not suppressed by ATP, despite the fact that under these conditions the insulin response is reduced by more than 50%. These results further emphasize that the specificity of the ATP effect is restricted to the insulin-controlled component of glucose transport.

**Effects of Phosphonic Acid Analogs of ATP**—The effects described above for ATP on insulin-stimulated glucose oxidation can be reproduced by using Ap(CHz)pp, an analog of ATP whose terminal phosphate group is available for hydrolytic and transfer reactions (Table V). Since this compound is not a substrate for adenylate cyclase (54), it is unlikely that the effects of ATP result from stimulation of this enzyme or from the production of cyclic AMP. Unlike ATP,

**Effects of ATP on Ouabain-stimulated Glucose Oxidation**—

<table>
<thead>
<tr>
<th>Addition</th>
<th>10 μM ATP</th>
<th>100 μM ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat germ agglutinin</td>
<td>None</td>
<td>12,900 ± 400</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>19,180 ± 400</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>76,800 ± 2,100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>121,700 ± 1,000</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>None</td>
<td>13,000 ± 400</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>91,100 ± 5,000</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>112,700 ± 9,000</td>
</tr>
</tbody>
</table>

* Conditions are as described in Fig. 1.

* Counts per min of 14CO2 production in 2 hours; average ± S.E.

**Effect of ATP on spermine and spermidine-stimulated rates of glucose oxidation by fat cells**

<table>
<thead>
<tr>
<th>Addition</th>
<th>10 μM ATP</th>
<th>100 μM ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermine</td>
<td>None</td>
<td>13,900 ± 500</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>14,500 ± 100</td>
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<tr>
<td></td>
<td>16</td>
<td>21,200 ± 400</td>
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<tr>
<td></td>
<td>160</td>
<td>35,700 ± 1,000</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>53,100 ± 200</td>
</tr>
<tr>
<td>Spermidine</td>
<td>None</td>
<td>11,100 ± 200</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>11,200 ± 300</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>20,100 ± 1,000</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>35,100 ± 1,800</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>34,200 ± 600</td>
</tr>
</tbody>
</table>

* Conditions are as described in Fig. 1.

* Counts per min of 14CO2 production in 2 hours; average ± S.E.
the analog, Ap(CH2)p, at high concentrations has effects on the ATP in the medium before measuring glucose oxidation. These results suggest that the effects of ATP on glucose transport are dependent on the hydrolysis or transfer of the terminal phosphate group of the nucleotide.

Furthermore, this compound, which is an inhibitor of phosphokinase reactions involving ATP (31, 55),5 protects the cells from processes unrelated to those under study since in cells treated with Ap(CH2)p and washed before testing, no effects on basal rates of glucose oxidation are detected (to be described shortly). The α,β-methylene analog of ADP, Ap(CH2)p, has no effects on basal or stimulated rates of glucose oxidation even at concentrations as high as 1 mM. The ATP analog, Ap(CH2)p, which cannot participate in hydrolytic or transfer reactions of its terminal phosphate moiety, does not significantly alter basal or insulin-stimulated glucose oxidation even at concentrations as high as $10^{-8}$ M (Table V). Furthermore, this compound, which is an inhibitor of phosphokinase reactions involving ATP (31, 55), protects the cells from the effects of Ap(CH2)p (Table V) and ATP (not shown). These results suggest that the effects of ATP on glucose transport are dependent on the hydrolysis or transfer of the terminal phosphate group of the nucleotide.

Irreversibility of ATP Effect—The studies described above suggested the possibility that the ATP effect on insulin-stimulated glucose transport could be occurring by phosphorylation of cell surface structures. Although the inherent instability and enzymic susceptibility of ATP to hydrolysis made it likely that its concentration would rapidly decrease during the period of incubation with cells, it was not known whether the continual presence of ATP in the incubation medium was needed for its effect on glucose transport to be manifest. Studies were therefore performed in which fat cells were incubated with ATP at 24°C for varying periods of time and washed thoroughly to remove the ATP in the medium before measuring glucose oxidation.

Exposure of fat cells (24°C) to low concentrations (5 to 50 μM) of ATP for periods as short as 2 to 5 min, followed by washing, results in a very significant suppression of insulin-stimulated glucose oxidation (Fig. 8). These effects are particularly striking when it is considered that following such mild treatment the cells are incubated at 37°C for 60 min for studying glucose oxidation. It is clear that transient exposure to ATP results in relatively permanent effects on the insulin response.

To examine in more detail the apparent irreversibility of exposure of cells to ATP, cells were incubated at 24° for 5 min with 10 and 100 μM ATP. After washing thoroughly, the cells were incubated at 37°C in the absence of glucose for 4 hours. These results suggest that the effects of ATP on glucose transport to be manifest. Studies were therefore performed in which fat cells were incubated with ATP at 24°C for varying periods of time and washed thoroughly to remove the ATP in the medium before examining glucose oxidation. Exposure of fat cells (24°C) to low concentrations (5 to 50 μM) of ATP for periods as short as 2 to 5 min, followed by washing, results in a very significant suppression of insulin-stimulated glucose oxidation (Fig. 8). These effects are particularly striking when it is considered that following such mild treatment the cells are incubated at 37°C for 60 min for studying glucose oxidation. It is clear that transient exposure to ATP results in relatively permanent effects on the insulin response.

Experiments similar to those described above were performed with some well known inhibitors of glucose transport to determine whether irreversible or residual inhibitory effects could be detected after briefly (5 min) treating the cells with these compounds followed by washing. Under these conditions no inhibitory effect was demonstrable with 2 mM phlorizin (14, 27), 1 mM phloretin (14, 27), 100 μM 3-O-methylglucose (14, 27), or 10 μg per ml of cytochalasin B (56-58) on insulin-stimulated rates of glucose oxidation.

Because of the results described above, most subsequent studies were performed by briefly incubating cells followed by washing to remove the ATP in the medium before examining the properties of glucose oxidation of the cells. Under these conditions interpretations are simpler because it is much less likely that complicating metabolic processes are occurring. By such procedures, treatment of cells with 1 μM cyclic AMP, AMP, adenosine, or with Ap(CH2)p do not modify basal or insulin-stimulated rates of glucose oxidation.

Protection from ATP Effect by Phloretin and Ap(CH2)p—Since the above studies suggested the possibility that ATP was phosphorylating and inactivating membrane structures specifically involved in glucose transport (carriers), studies were performed to determine whether competitive inhibitors of glucose transport, which presumably share the same carrier as glucose, could modify the effects of ATP. Phlorizin, a potent competitive inhibitor of n-glucose transport (14, 27, 30, 59) in fat cells, effectively blocks the effect of ATP when it is added to fat cells 5 min before the addition of ATP (Table VI).6 Phlorizin, a polyphenolic-β-glucoside which also inhibits n-glucose transport (in fat cells) but with a 50-fold lower affinity ($K_i = 2.4 \times 10^{-4}$ M, Ref. 17), does not protect but instead potentiates the effect of ATP.

Under these conditions the β,γ-methylene derivative of ATP, Ap(CH2)p, has at high concentrations has effects on the ATP in the medium before measuring glucose oxidation. These results suggest that the effects of ATP on glucose transport are dependent on the hydrolysis or transfer of the terminal phosphate group of the nucleotide.

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Addition | 
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
</tr>
<tr>
<td>None, ..........................</td>
</tr>
<tr>
<td>App(CH$_2$)$_2$p (0.65 mM)</td>
</tr>
<tr>
<td>Insulin (10 microunits/ml)</td>
</tr>
<tr>
<td>App(CH$_2$)$_2$p (0.65 mM) + insulin (10 microunits/ml)</td>
</tr>
</tbody>
</table>

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* Conditions are as described in Fig. 1.

* Counts per min of $^{14}$C$_2$O$_4$ production in 2 hours; average ± S.E.

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**FIG. 8.** Effect of preincubating fat cells with ATP (5 $\times$ 10$^{-6}$ M; O; 5 $\times$ 10$^{-4}$ M, ▲) for varying time periods on the insulin-stimulated conversion of $\nu$-[U-$^{14}$C]glucose to $^{14}$C$_2$O$_4$ by isolated fat cells. The fat cells were preincubated with ATP at 24°C in Krebs-Ringer bicarbonate buffer containing 1% albumin for time periods indicated. The reaction was stopped by the addition of a 30-fold excess of the same buffer followed by washing three times. The cells were then incubated for 1 hour at 37°C with $\nu$-[U-$^{14}$C]glucose (0.2 mM, 0.5 μCi per μmole) and insulin (100 microunits per ml) in the same buffer. The results are expressed as per cent of the insulin response observed in cells treated similarly but without ATP.

App(CH$_2$)$_2$p, completely protects against the action of ATP (Table VI).

A number of other compounds that are known to inhibit glucose transport or to share the same transport mechanisms, such as cytochalasin B (10 μg per ml), 3-O-methylglucose (100 mM) and γ-glucose (20 mM) do not protect from the effect of ATP. It is not too surprising that the simple sugars cannot effectively compete with the probably covalent reaction of ATP because of the very low affinity (near 1 mM) of these sugars for the transport system. The inhibition is also unaffected by insulin (240 microunits per ml, ▲), cyclic AMP (0.2 mM, ▲), cyclic GMP (0.2 mM, ▲), or by high concentrations (1 mM) of the nucleoside triphosphates GTP, ITP, CTP, and UTP.

**Effect of ATP on Antilipolytic Activity of Insulin**—Treatment of fat cells with ATP, followed by washing of the cells, does not modify basal or norepinephrine-stimulated rates of lipolysis of these cells, and the antilipolytic activity of insulin is unaltered (Table VII). These results indicate that ATP does not generally suppress all of the metabolic effects of insulin, which is consistent with the results described earlier which suggest that ATP selectively modifies specific components of the glucose transport system which are activated by insulin.

**Studies of 3-O-Methyl-$\nu$-glucose Transport**—Although the above data suggest that exogenous ATP is altering glucose oxidation by affecting glucose transport, more conclusive evidence was obtained by direct measurements of uptake of the nonmetabolized analog of γ-glucose, 3-O-methyl-$\nu$-glucose.

Because 3-O-methyl-$\nu$-glucose is not phosphorylated or metabolized by fat cells (14), and because of the very small intracellular cytoplasmic volume of these cells (38), only small amounts (about 0.5% of the sugar are taken up by the cells at equilibrium (Fig. 10). Furthermore, it is very difficult to measure initial rates of uptake because equilibration between the extra- and intracellular spaces occurs extremely rapidly. These difficulties can now be partially overcome by the technique of oil-flotation separation (38), which permits separation of the
Protection from ATP suppression of insulin-stimulated glucose oxidation by phloretin and App(CH$_2$)$_6$P

Fat cells were incubated with protective agents for 5 min at 24°C in Krebs-Ringer-bicarbonate buffer containing 1% albumin before the addition of ATP. The cells were washed four times with 10 times the original incubation volume of the above buffer 5 min after the addition of ATP. The cells were then suspended in the same buffer and incubated with 0.2 mM $\beta$-[U-14C]glucose (5 $\mu$Ci per pmole) in the absence and presence of 100 microunits per ml of insulin. The cell concentration was about 2 $\times$ 10$^6$ cells per ml.

**TABLE VI**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>$^{14}$CO$_2$ production$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal activity (100 microunits/ml)</td>
</tr>
<tr>
<td>None</td>
<td>8,900 ± 100</td>
</tr>
<tr>
<td>Phloretin (1 mM)</td>
<td>7,900 ± 100</td>
</tr>
<tr>
<td>ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>8,100 ± 200</td>
</tr>
<tr>
<td>Phloretin (1 mM) + ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>17,700 ± 400</td>
</tr>
<tr>
<td>None</td>
<td>9,600 ± 300</td>
</tr>
<tr>
<td>Phloretin (1 mM)</td>
<td>10,600 ± 100</td>
</tr>
<tr>
<td>ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>7,700 ± 200</td>
</tr>
<tr>
<td>Phloretin (1 mM) + ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>9,300 ± 200</td>
</tr>
<tr>
<td>App(CH$_2$)$_6$P (2 mM)</td>
<td>10,200 ± 400</td>
</tr>
<tr>
<td>ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>8,000 ± 200</td>
</tr>
<tr>
<td>App(CH$_2$)$_6$P (2 mM) + ATP (2 $\times$ 10$^{-4}$ M)</td>
<td>10,200 ± 100</td>
</tr>
</tbody>
</table>

* Counts per min of $^{14}$CO$_2$ production in 1 hour; average ± S.E.

**TABLE VII**

Effect of incubating fat cells with ATP on antilipolytic activity of insulin

Fat cells (about 2 $\times$ 10$^6$ cells per ml) were incubated for 10 min at 24°C in Krebs-Ringer bicarbonate buffer containing 1% albumin and the indicated concentration of ATP. The cells were washed four times with 5 times the original incubation volume of the above buffer containing no ATP. The cells were then suspended in Krebs-Ringer bicarbonate buffer containing 3% albumin and incubated at 37°C for 80 min in the absence and presence of insulin (90 microunits per ml), L-norepinephrine (80 ng per ml) or both.

<table>
<thead>
<tr>
<th>ATP concentration</th>
<th>Glycerol released$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No additions</td>
</tr>
<tr>
<td>$^n$</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>10$^{-5}$</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>5 $\times$ 10$^{-4}$</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>5 $\times$ 10$^{-4}$</td>
<td>4.6 ± 0.5</td>
</tr>
</tbody>
</table>

* Micromoles per mmole of cell triglyceride, average value ± S.E.

Insulin reproducibly stimulates 3-O-methyl-d-glucose uptake (Fig. 10). Although the uptake of this sugar by fat cells is very fast, the enhancement by insulin is marked in the presence of the hormone uptake is already saturated by 20 s. The uptake of 3-O-methyl-d-glucose in the presence of insulin is consistently higher at 20 s than at 45, 60, or 120 s. This may be due to a stimulation of efflux as well as influx by insulin. ATP at 5 $\times$ 10$^{-5}$ M reproducibly and significantly decreases the insulin-stimulated transport of 3-O-methyl-d-glucose (Fig. 10). Paired analysis ($t$ test) from three separate experiments shows $t = 23.8$, $p > 0.01$, for this effect. It is likely that the real effect of ATP is even greater than that suggested by the data in Fig. 10 since, whereas the insulin-stimulated uptake is already maximal at 20 s, saturation requires longer time if the nucleotide is present. The initial rate of uptake in the presence of insulin is clearly much faster than can be measured by the present techniques. ATP does not depress the rate of uptake in the absence of insulin. Although the data in Fig. 10 suggest that ATP may stimulate the rate of uptake of 3-O-methyl-d-glucose in the absence of insulin, this effect is not reproducible in separate experiments. This differential effect of ATP on basal and insulin-stimulated uptake is especially significant in view of the fact that the much slower rate of basal (unstimulated) uptake permits more accurate measurements than in the presence of insulin.

Phloretin (1 mM) inhibits nearly completely the basal as well as the insulin-stimulated uptake (Fig. 10). Insulin effects can, however, be detected even in the presence of 1 mM phloretin. The effects of ATP on the insulin-stimulated uptake of 3-O-methyl-d-glucose uptake confirm the data obtained by measurements of glucose oxidation.

Phosphorylation of Membrane Components with $^{32}$P—Isolated intact fat cells were incubated with $[\gamma ^{32}$P]ATP of very high concentration.
isolated fat cells by incubating with 5 pmol of fat cell membrane phosphorylated in intact cells containing 1% albumin. The reaction was stopped by the addition of 10 times the original incubation volume and washing three times. Purification of the plasma membrane and the incubation procedures were described under "Materials and Methods." Phloretin (1 mM) and App(CH2)p (1 mM) were added 5 min before the addition of [γ-32P]ATP.

specific activity for 5 min at 24°C, and the cells were washed thoroughly. The microsomal membrane, mitochondrial and nuclear fractions were subsequently purified as described under "Materials and Methods." Each of these fractions was solubilized and subjected to sodium dodecyl sulfate gel electrophoresis, and the distribution of 32P on the gels was determined. Although no radioactive peaks are detected in the material derived from the nuclear and mitochondrial fractions, two principal, closely spaced peaks are evident in the material containing plasma membranes (Fig. 11). These peaks correspond to molecular weights of about 16,000 and 22,000.

When the phosphorylation reaction with intact cells is performed in the presence of phloretin (1 mM), the radioactive peak corresponding to molecular weight 22,000 is substantially decreased (Fig. 11). Both 32P-labeled peaks are diminished if the phosphorylation reaction is performed in the presence of 1 mM App(CH2)p. There is no evidence that significant phosphorylation of membrane phospholipids occurs under the conditions of these studies.

**DISCUSSION**

The inhibition by ATP of insulin-stimulated glucose oxidation appears to represent a selective suppression of the specific glucose transport mechanism which is normally activated by this hormone. It is very unlikely that such treatment results in interference with intracellular metabolic events. Glucose oxidation is measured under conditions where glucose transport is probably the rate-limiting step in the total pathway for the oxidation of glucose (13, 14, 16, 18). The effects of insulin on stimulating basal glucose oxidation are normally mediated by increasing the rate of transport of this sugar (13, 14). ATP treatment specifically inhibits the insulin-stimulated but not the basal rates of glucose oxidation, and the inhibition is not demonstrable at high glucose concentrations where transport processes are no longer rate-limiting. Furthermore, ATP is effective at concentrations 2 to 3 orders of magnitude lower than the known intracellular concentrations of ATP (43, 44), and it is known that the fat cell membrane is relatively impermeable to ATP (60). Despite the inherent instability of ATP and its susceptibility to tissue ATPases, the inhibition is effective after exposing the cells to ATP for only a few minutes, and the effect persists after removal of the ATP in the medium.

The data indicate that ATP treatment decreases relatively specifically the apparent affinity of the membrane carrier system for D-glucose, and it has no effect on the maximal rates of glucose transport or oxidation. This effect, furthermore, is demonstrable only in the presence of insulin or other insulin-like substances (wheat germ agglutinin, concanavalin A, thiol compounds, and polyamines) that appear to activate the same glucose transport system as insulin. The effect of ouabain on glucose oxidation, which appears to be mediated by a mechanism different from that of insulin (Fig. 6) is not altered by ATP treatment of the cells (Fig. 7). The ATP effect thus appears to represent a very selective modification of a specific property (apparent affinity for glucose) of that portion of the transport system which is subject to regulation by insulin. Since normally the predominant effect of insulin is to increase the apparent affinity of the transport system for glucose (14), ATP behaves as a relatively specific antagonist of insulin action on glucose transport. The suggestion that exogenous ATP modulates glucose transport processes is confirmed by the demonstration that this nucleotide can inhibit the insulin-stimulated but not the basal rate of uptake of D-3-O-methyl-D-glucose in isolated fat cells (Fig. 10). These results suggest the possibility that the mechanism by which ATP treatment modifies glucose transport may potentially serve as a physiological means of regulating a specific effect of insulin on adipose (and perhaps other) tissue.

ATP does not appear to act as a general antagonist of insulin action since treatment of cells with ATP does not alter the antilipolytic activity of the hormone (Table VII). Treatment of cells with ATP does not modify the apparent affinity of the insulin-receptor interaction, as judged from dose-response relationships in studies of glucose transport (Fig. 2) and of lipolysis (Table VII), as well as from direct measurements of 125I-insulin binding (Table I). These considerations, together with the effect of ATP on insulin-like substances (thiols and polyamines) which do not act by activating insulin receptors, suggest that the effect of ATP is at a step distal to the initial receptor interaction. ATP treatment of cells is thus an effective means of dissociating the action of insulin on glucose transport from other actions such as its antilipolytic activity. The results, therefore, support the view that the effects of insulin on transport processes are not responsible for all of the intracellular effects of the hormone. Furthermore, such considerations imply that enhancement of transport by insulin may not be an integral function of the receptor macromolecules themselves. It is probably more likely that transport is a distal process similar to other intracellular biochemical processes which are subject to regulation by chemical mediators originating at the level of the cell membrane.

The fact that App(CH2)p mimics the effect of ATP while
App(CH2)p actually protects from this effect and Ap(CH2)p is without effect, coupled with the relatively irreversible nature of the inhibition that follows a brief exposure to ATP, suggest that the inhibition is mediated by a phosphorylation reaction involving the γ-phosphate moiety of ATP. This very low concentration of exogenous ATP which is required, especially relative to the intracellular concentration of the nucleotide, the facile nature of the reaction, the impermeability of the cell membrane for ATP (60), and the fact that the effect of ATP appears to be restricted to a relatively specific membrane-localized function suggest strongly that such phosphorylation is occurring at the level of the cell membrane. The present studies demonstrate directly that restricted and apparently specific phosphorylation of components of the cytoplasmic membrane indeed occurs by such mild treatment with [γ-32P]ATP (Fig. 11). Furthermore, App(CH2)p inhibits this phosphorylation, and the competitive antagonist of glucose transport, phloretin, can selectively suppress the phosphorylation of one of the two labeled components of the membrane. These results correlate well with the ability of phloretin and App(CH2)p to protect from the irreversible biological effects of ATP treatment.

These results, particularly in view of the modification of both the biological effects and the phosphorylation reaction by competitive antagonists of glucose transport (phloretin and phlorizin), suggest that the phosphorylation reaction involves membrane structures concerned with glucose transport (carriers). These studies thus suggest a possible means of examining by chemical methods hormone-sensitive transport processes in mammalian cells. Studies are in progress to examine in detail and to isolate and purify the two phosphorylated membrane components, particularly the one having a molecular weight of about 22,000.

The regulatory function of phosphorylation and dephosphorylation reactions in a variety of enzyme systems has been well documented (32). Because of the possible involvement of cyclic AMP or cyclic GMP in at least certain actions of insulin, it may be of particular interest that cyclic AMP-dependent protein kinases and phosphatases localized in cell membranes have been described in many tissues. Weller and Rodnitzky (61) have described protein kinase activity in ox brain microsomal and synaptosomal preparations. Majumber and Turkington (62) recently demonstrated hormonal dependent phosphorylation of plasma membrane proteins of the mouse mammary gland in vitro. Permeability changes mediated by membrane phosphorylation have been implicated in the toad bladder (63), and phosphoprotein kinases dependent on cyclic AMP exist in rat cerebral synaptic membranes (64). Cyclic AMP-stimulated phosphorylation of erythrocyte ghosts has been described (65, 66). Dousa et al. (67) reported cyclic AMP-dependent phosphorylation of renal medullary plasma membrane protein. Recently Delorenzo and Greengard (68) have described the activation by cyclic AMP of a membrane-bound phosphoprotein phosphatase in toad bladder.

Although the ATP inhibition of glucose transport could not be reversed by insulin or by glucose, the possibility that the phosphorylation described in these studies may play an important regulatory function is intriguing. It has been shown that in purified fat cell plasma membranes, minimal phosphorylation occurs by incubation with [γ-32P]ATP alone, but that cyclic AMP markedly catalyzes the phosphorylation of the two membrane components described in this report. The possibility exists that insulin may by this mechanism exert an in vivo regulatory function on transport by virtue of its ability to decrease intracellular levels of cyclic AMP (2-0) or perhaps more directly by its inhibition of the membrane-localized activities of adenylyl cyclase (5-12) or phosphodiesterase (60, 70). Because of the localization of all of these elements to the cell membrane, the local concentration (or turnover) of cyclic AMP in the immediate environment of the membrane may be more important in this function than the total cell level of the nucleotide.

Because of the relatively protracted and irreversible nature of the inhibitory effect of ATP on the insulin-stimulated rates of glucose transport, it may be worthwhile considering the possibility that these mechanisms may play a more important role in long range, adaptive regulation of insulin sensitivity. It will be interesting, for example, to determine whether in certain metabolic states, such as protracted hyperglycemia, diabetes, corticosteroid administration, and other insulin-resistant states, the specific membrane component discussed above exists in a relatively phosphorylated state, thus possibly accounting at least in part for insulin insensitivity in peripheral tissues.

It is notable that exogenous ATP is effective at concentrations very far below those that normally exist in the cell, and that the phosphorylation reaction occurs so quickly (5 min) and under very mild conditions (24°). This suggests that the phosphorylation reaction is occurring in a relatively superficial region of the membrane, and that this site is more accessible to exogenous compared to endogenous ATP. If this phosphorylation reaction plays an important physiological role in the regulation of insulin-stimulated glucose transport, modulation of the phosphorylation reaction may depend critically on the regulation of the mechanisms by which cytoplasmic ATP is delivered to the enzyme, the localized generation of ATP by membrane-localized enzymes, or the concentration of ATP in the serum and tissue fluids.

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Adenosine Triphosphate-dependent Inhibition of Insulin-stimulated Glucose Transport in Fat Cells: POSSIBLE ROLE OF MEMBRANE PHOSPHORYLATION
Kwen-Jen Chang and Pedro Cuatrecasas


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