Cyclic Adenosine Monophosphate-dependent Phosphorylation of Specific Fat Cell Membrane Proteins by an Endogenous Membrane-bound Protein Kinase

POSSIBLE INVOLVEMENT IN THE REGULATION OF INSULIN-STIMULATED GLUCOSE TRANSPORT*

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SUMMARY

The phosphorylation of specific membrane proteins by an endogenous protein kinase has been studied in purified membrane fractions from rat adipocytes using trichloroacetic acid precipitation and sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. The endogenous phosphorylation of two specific membrane proteins is completely dependent on the presence of cyclic adenosine 3’:5’-monophosphate (cyclic AMP) and magnesium ions. This phosphorylation occurs very rapidly at 24°, reaching maximal levels at 1 min. The number of sites specifically phosphorylated in the presence of cyclic AMP probably does not exceed 50,000 per fat cell, requiring the use of very high specific activity (10 to 50 Ci per mmole) [γ-32P]ATP for these studies.

The minimal molecular weights of the two specifically phosphorylated proteins are about 22,000 and 16,000 as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The same two proteins are phosphorylated when intact fat cells are exposed briefly to low concentrations of exogenous ATP, a process which results in the suppression of the insulin-stimulated rates of D-glucose transport. At least 95% of the cyclic AMP-dependent 32P incorporated into trichloroacetic acid-precipitable protein is in the form of protein-bound phosphoserine. The concentration of cyclic AMP required for maximal stimulation of phosphorylation is about 1.5 μM. Cyclic guanosine 3’:5’-monophosphate has no significant effect unless its concentration is increased to 10–4 M. Phosphorylation is inhibited by calcium ions. The cyclic AMP-stimulated phosphorylation is inhibited by phloretin and by 5’-adenyl-β,γ-methylene triphosphonate.

The specific cyclic AMP-dependent membrane phosphorylation is also found in membranes from cells of obese rats, an insulin-resistant animal. This phosphorylation system, however, cannot be detected in membranes from guinea pig fat cells. The lack of phosphorylation of specific membrane proteins in guinea pig fat cell membranes is correlated with the insulin insensitivity of this tissue to glucose transport (but not lipolysis) and with the inability of ATP to inhibit the slight stimulation which insulin exerts on glucose transport. The possibility is considered that this specific cyclic AMP-dependent phosphorylating system is involved in the hormonal regulation of glucose transport and the modulation of insulin sensitivity.

The regulatory function of phosphorylation and dephosphorylation reactions in a variety of soluble enzyme systems has been well documented, and the importance of cyclic adenosine 3’:5’-monophosphate in these processes has been studied in detail (1, 2). The view was advanced (3, 4) some time ago that the diverse effects of cyclic AMP may be mediated through regulatory protein kinases, specificity being achieved in given tissues according to the nature and localization of the kinases as well as the specific substrates. Cyclic AMP-dependent protein kinases and phosphatases localized in subcellular membrane fractions have been described in many tissues, and the participation of membrane phosphorylation has been implicated in the regulation of synaptic transmission, binding of ions, and membrane permeability. Endogenous cyclic AMP-dependent protein kinases which phosphorylate endogenous membrane proteins have been described in brain membrane fragments and synaptic preparations (5–8), human erythrocyte ghosts (9–12), bovine anterior pituitary particulate fractions (13, 14), and

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1 The abbreviations used are: cyclic AMP, cyclic adenosine 3’:5’-monophosphate; cyclic GMP, cyclic guanosine 3’:5’-monophosphate; App(CH,)</ref>·P, 5’-adenyl-β,γ-methylene triphosphonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N’,N’-tetraacetic acid.
membrane preparations from mouse mammary gland (15), renal medulla (16) and cortex (17), testis (18), canine heart (19), adrenal gland (20), and myelin basic protein (21). De Lorenzo and Greengard (22) have implicated a cyclic AMP-stimulated, membrane-bound phosphoprotein phosphatase in the action of hormonally (antidiuretic hormone) mediated permeability changes in the toad bladder. Andrew et al. (23) recently described the endogenous phosphorylation of three proteins of very low molecular weight (less than 30,000) in muscle membranes, but cyclic AMP was virtually without effect on this process.

The diverse effect of many hormones may be mediated by changes in the intracellular levels of cyclic AMP (24). Insulin under certain circumstances may alter the intracellular concentrations of cyclic AMP (25-30), perhaps by inhibiting directly the membrane-localized activity of adenylate cyclase (31-37) or by activating cyclic AMP phosphodiesterases (38-40). The recent demonstration that insulin increases the intracellular levels of cyclic GMP (41) in adipocytes and liver slices also suggests a possible role of this cyclic nucleotide in the mediation of insulin responses. Although certain metabolic effects of insulin, such as those observed on lipolysis and glycogen synthesis, could perhaps be explained by effects of known cyclic AMP-dependent protein kinases, it has not been possible to explain the effects of insulin on glucose transport by invoking cyclic AMP-mediated processes. Despite demonstrations that cyclic AMP and related analogs can modify glucose oxidation and transport processes in rat adipocytes (42-46), simple interpretations of these studies are difficult (47) and the possible relationship between cyclic nucleotides and glucose transport remains elusive. Attempts to relate the effects of insulin or of cyclic AMP on glucose transport to possible phosphorylation reactions in the cell membrane have heretofore not been described.

We have recently demonstrated that exogenous ATP at low concentrations (less than 10^{-3} M) inhibits quickly and irreversibly insulin-stimulated glucose transport in isolated rat fat cells (48). This effect is specific for insulin-stimulated glucose transport and does not alter the antilipolytic activity of insulin. This inhibitory effect is well correlated with the phosphorylation of two specific membrane components which are detected by subfractionation of intact, $[gamma]^{32}$P]ATP-treated cells. Phoretin, a competitive antagonist of glucose transport, and the phospho-

**MATERIALS AND METHODS**

**Materials**—Cyclic AMP, cyclic GMP, and nucleotide triphosphates were purchased from Sigma. Phoretin was purchased from K & K Laboratory. Appo (CH$_3$)$_2$ was purchased from P-L Biochemicals. 3-O-Methyl-d-[methyl-$^3$H]glucose (specific activity, 1.2 Ci per mmole) was purchased from ICN. Staphylococcal nuclease and bovine pancreatic trypsin were from Worthington, pronase from Calbiochem, and Clostridium perfringens phospholipase C from Nutritional Biochemicals.

The [gamma]ATP was synthesized by the method of Glynn and Cappell (48). The reaction is carried out at about 24° in the lead glass bottle in which the [gamma]P (New England Nuclear, 10 to 30 mCi in 1 ml of 0.02 N HCl) is received. HCl is neutralized by Tris base. The reaction mixture (1.1 ml), which contains 2 mmoles of MgCl$_2$, 1 m mole of 3-mercaptoethanol, 0.5 m mole of EDTA, 0.5 m mole of 3-phosphoglycerate, 0.05 m mole of NAD$^+$, and 0.2 m mole of ATP, is adjusted to pH 8.0 with Tris base. The reaction is initiated by adding 10 [micrograms] of 3-phosphoglycerate kinase (Boehringer) and 30 [micrograms] of glyceraldehyde phosphate dehydrogenase (Boehringer). After incubation for 30 minutes under these conditions, the reaction is stopped by boiling for 1 minute. The labeled ATP is separated from the other components of the reaction mixture by column chromatography on DEAE-Sephadex A-25 (Pharmacia) using gradient elution consisting of 200 ml of H$_2$O and 200 ml of 1 M triethylamine bicarbonate, pH 8.3. The fractions containing significant amounts of ATP are pooled, desalted by evaporation to dryness in an evaporator, rinsed and evaporated twice with absolute ethanol, and stored at −20° in a small volume of double distilled water. The recovery of $[gamma]^{32}$P]ATP from [gamma]$^3$P is about 80%. The purity of the $[gamma]^{32}$P-labeled ATP product is checked by thin layer chromatography on PEI (polyethyleneimine)-cellulose (J. T. Baker Chemical Co.) in 0.2 M ammonium bicarbonate. The purity is greater than 95% and the specific activity is about 40 to 50 Ci per mmole.

**Preparation of Fat Cell Membrane Fraction**—Isolated fat cells were prepared from about 150-g male Sprague-Dawley rats according to the method of Rodbell (49). Membranes were purified by a modification of the method of Avaru and Wallach (50). Isolated fat cells (from about 10 rats) are suspended in 10 ml of ice-cold homogenization buffer containing 50 ml of sucrose, 1 M isonicotic acid, 10 m moles of Na$_2$EDTA, pH 7.4. The cells are homogenized with a Polytron PT-10 (Brinkmann) at a speed set at 2.5 for 30 s. The total particulate fraction is obtained by centrifugation at 40,000 × g for 30 min. The pellet, suspended in 10 ml of the same buffer, is layered on 35% sucrose containing 10 m moles of Na$_2$EDTA, pH 7.4, and centrifuged in a Beckman SW 27 rotor at a speed of 25,000 rpm (4°) for 60 min in a Spinco model L2-65B ultracentrifuge. The membrane material located in the interface is aspirated and diluted with 6 volumes of buffer containing 50 m moles sodium acetate, 1 m mole magnesium acetate, and 0.3 m moles EGTA, pH 6.0. The suspension is centrifuged at 40,000 × g for 30 min, and the pellets are immediately reassembled in the same buffer for measurement of protein kinase activity. The yield of cytoplasmic membrane obtained by these fractionation procedures can be estimated accurately and by briefly exposing the intact cells to $[gamma]^{32}$P-labeled wheat germ agglutinin (51) or $[gamma]^{32}$P-labeled insulin (52) followed by cooling to 4° and washing of the cells before homogenization (53). The labeled compounds used are very specific plasma membrane markers which exhibit virtually no dissociation (51, 52) during the time periods required by these procedures. By this method the percentage of cytoplasmic membrane obtained from intact cells is about 40%.

**Standard Protein Kinase Assay**—Protein kinase activity is measured in sodium acetate buffer, pH 6.0, in a final volume of 50 ml containing 50 m moles sodium acetate, 1 m mole magnesium acetate, 2 m moles ammonium phosphate, 0.3 m moles EGTA, 1 m mole sodium fluoride, 20 m moles $[gamma]^{32}$P]ATP (10 to 50 Ci per mmole), and 10 to 50 m moles of purified membrane protein. The incubation mixture is dialyzed at pH 6.0 with or without cyclic AMP at 24° for 20 min before initiating the reaction by the addition of $[gamma]^{32}$P]ATP. In the standard assay the mixture is incubated at 24° for 1 min, and the reaction is terminated by adding in rapid succession 5 ml of 10% bovine serum albumin and 2 ml of ice-cold trichloroacetic acid containing 0.1 m mole of phosphoric acid. A 4° centrifugation at 4° for 15 min is carried out, and the supernatant is centrifuged in an International refrigerated centrifuge at a speed of 3000 rpm. The supernatant is decanted, and the pellet is dissolved in 0.2 ml of 1 N NaOH and precipitated again with 2 ml of ice-cold 10% trichloroacetic acid containing 0.1 m mole of phosphoric acid. This step is repeated twice. The final, well drained pellet containing 0.2 ml of 1 N NaOH and the reaction mixture is deionized in 13 ml of Bray's solution and counted in solution (54). The data are expressed as picomoles of $[gamma]^{32}$P phosphate incorporated per mg of protein added. All assays are performed in duplicate. The protein concentration is determined by the method of Lowry et al. (55) after heating at 100° in 1 N NaOH for 30 min; crystalline bovine serum albumin is used as the standard.

**Polyacrylamide Disc Gel Electrophoresis**—Phosphorylated membrane proteins are prepared as described for the standard kinase assay except that the reaction is stopped by adding 5 ml of 10% sodium dodecyl sulfate. The samples containing sodium dodecyl sulfate

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are incubated at 37° for 30 min in 0.1 m phosphate buffer, pH 7.0, containing 3 mm dithiothreitol and 25% (w/v) sucrose (56). The membranes, which appear to be completely solubilized, are subjected to electrophoresis in 10% polyacrylamide disc gels containing 0.1% sodium dodecyl sulfate and 0.1 m sodium phosphate buffer, pH 7.0 (56). Electrophoresis is performed with a current of 5.5 ma per tube for 4½ hours, using cytchrome c, bovine serum albumin, and ovalbumin as standards. Proteins are detected by staining overnight with Coomassie blue (0.05% in 25% isopropyl alcohol, 10% acetic acid), and the gels are de-stained for 6 to 7 hours with diluted Coomassie blue (0.0025% in 10% isopropyl alcohol, 10% acetic acid) followed by washing with 10% acetic acid until the background becomes clear (57).

The gels are sectioned (about 1.2 mm) with a manual slicing device and radioactivity is determined after dissolving the gel samples by incubating in 0.1 ml of 50% H2O2 for 24 hours at 24°. The dissolved gels are counted in Bray’s scintillation fluor.

Hydrolysis and Electrophoresis of 32P Material in Membrane—

The 32P-phosphorylated membrane material, precipitated with 10% trichloroacetic acid containing 0.1 m phosphoric acid, is washed three times as described for the standard protein kinase assay. The final, well drained pellets are hydrolyzed in 1 ml of 2 N or 6 N HCl at 105° for 4 hours in tubes sealed under vacuum. The hydrolysates are dried by lyophilization, dissolved in 100 ml of H2O, and subjected to electrophoresis on Whatman No. 3MM paper for 1 hour at 2500 volts and at pH 1.9 (2.5% formic acid, 7.8% acetic acid). [32P]Orthophosphate, phosphoserine, and phosphothreonine are used as markers. Amino acids are visualized with ninhydrin spray, and radioactivity is located by cutting the chromatogram and counting in Bray’s scintillation fluor.

RESULTS

Time course of Phosphorylation in Presence and Absence of Cyclic AMP—Incubation of fat cell membranes with [γ-32P]ATP at 24° results in the incorporation of trichloroacetic acid-precipitable radioactivity. Under the standard assay conditions, phosphorylation of the membrane occurs very rapidly, reaching a maximal level within 1 min (Fig. 1). Cyclic AMP (1.5 μM) exerts a stimulatory effect at all incubation times tested. Measurements of the amount of [γ-32P]ATP hydrolyzed in the presence of 1.5 μM cyclic AMP reveal that 75% of the [γ-32P]ATP is hydrolyzed within 2 min, indicating that the concentration of [γ-32P]ATP probably becomes limiting after a 2-min period of incubation. Therefore a 1-min period of incubation was chosen for the standard assay procedure.

Specific Membrane Proteins Are Phosphorylated Only in Presence of Cyclic AMP—Cyclic AMP consistently stimulates the extent but perhaps not the rate of phosphate incorporation from [γ-32P]ATP into the membrane preparation. This type of stimulation suggested the possibility that cyclic AMP was facilitating the phosphorylation of specific membrane substrates which may not have been susceptible to phosphorylation in the absence of cyclic AMP. To examine the qualitative pattern of phosphorylation, purified membranes, phosphorylated with [γ-32P]ATP in the presence and absence of cyclic AMP (1.5 μM), were solubilized by treating with 1% sodium dodecyl sulfate-3 mm dithiothreitol. The individual polypeptides were resolved by disc gel electrophoresis in sodium dodecyl sulfate and stained by Coomassie blue (Fig. 2).

The pattern of protein bands in the disc gels resembles that recently described by Czech and Lynn (66). Identical protein patterns are obtained from membranes treated in the absence and presence of ATP, and no differences can be detected after incubating with cyclic AMP.

Virtually no radioactivity is detected in the disc gels when sodium dodecyl sulfate and [γ-32P]ATP are added simultaneously to the membranes in the presence or absence of cyclic AMP.
Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of 50 μg of purified fat cell membrane. The proteins were visualized by staining with Coomassie blue, and molecular weights were estimated by comparisons with the standard proteins, cytochrome c, bovine serum albumin, and ovalbumin. The corresponding [32P]phosphate peaks obtained by incubating the membranes with [γ-32P]ATP and cyclic AMP (shown in Fig. 3) are labeled by Peaks I and II. These radioactive peaks are not present if cyclic AMP is omitted from the phosphorylation reaction. Membrane phospholipids are visualized as an opaque, whitish band which migrates just behind the dye front and in the front of cytochrome c.

The two radioactive peaks visualized on disc gels do not correspond to any specific Coomassie blue-stained protein bands. Since about 2 pmoles of 32P are incorporated per mg of membrane protein, it can be calculated (assuming a molecular weight of 22,000) that 1 mg of membrane protein contains about 40 ng of Peak I protein. Since the total amount of membrane protein subjected to electrophoresis is only about 50 to 100 μg, it is not expected that the minute quantity of Peak I protein applied on the gel can be detected by protein stains. Similar considerations apply to Peak II protein.

Since the gel electrophoresis experiments show that these two 32P-phosphorylated peaks are absolutely dependent on the presence of cyclic AMP, and since no other specific proteins can be detected to be specifically phosphorylated in the absence of cyclic AMP, only those components showing cyclic AMP dependency will be characterized further.

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of material obtained from 50 μg of fat cell membrane phosphorylated for 1 min (24°) in the absence (B) and presence (C) of 1.5 μM cyclic AMP. The 0 min control (A) describes a reaction which was initiated by adding simultaneously 1% sodium dodecyl sulfate and [γ-32P]ATP in the presence of 1.5 μM cyclic AMP. The effect of phloretin (1 mM) on the cyclic AMP (1.5 μM)-dependent phosphorylation is also shown (D). Incubation conditions were as described in Fig. 1 except that the reaction was stopped by adding 1% sodium dodecyl sulfate.

Effect of Membrane Protein and ATP Concentration on Phosphorylation—The endogenous phosphorylation of the fat cell membrane protein is nearly proportional to the amount of purified membrane protein added up to 60 μg (Fig. 4). It is important to note that the total amount of [32P]phosphate incorporated in all of these experiments is quite small. Accurate detection and measurement requires that the substrate used, [γ-32P]ATP, be of very high specific activity (10 to 50 Ci per mmole).

The effect of cyclic AMP on the endogenous phosphorylation of fat cell membranes as a function of the concentration of ATP is shown in Fig. 5. Cyclic AMP enhances phosphorylation at all concentrations of ATP tested. Since the gel electrophoresis experiments demonstrate that phosphorylation of specific components is completely dependent on cyclic AMP, it is this portion of the total phosphorylation reaction which is of special interest. It can be estimated that the concentration of ATP required for half-maximal cyclic AMP-dependent phosphorylation is about 40 μM. It must be emphasized that this value for ATP and for
FIG. 4 (left). Effect of increasing the concentration of fat cell membrane protein on the amount of \[^{32}P\]phosphate incorporated into fat cell membrane proteins in the absence (O) and presence (O) of 1.5 μM cyclic AMP. The incubation conditions were as described in Fig. 1 except for the variation of fat cell membrane protein concentration. The samples were incubated for 1 min.

FIG. 5 (center). Effect of varying the concentration of ATP on the endogenous phosphorylation of fat cell membranes in the absence (A, O) and presence (A, O) of 1.5 μM cyclic AMP. Incubation (1 min) conditions were as described in Fig. 1 except the Mg\(^{2+}\), given below, should not be equated with a \(K_m\) value since the latter cannot be measured under our standard assay conditions because of the rapid rate of phosphorylation (prohibiting measurements of initial velocities) and the heterogeneity of substrates. Moreover, the presence in the membrane preparations of interfering enzymes such as ATPases and protein phosphatases further complicates precise kinetic or quantitative interpretation of the data. In the presence of very high, “saturating” concentrations of \([γ-\text{ATP}]\) (e.g. 0.1 mM), disc gel electrophoresis demonstrates very much greater radioactivity throughout the gel. Furthermore, under these conditions cyclic AMP does not very substantially increase the size of Peaks I and II (compared to experiments done with 10 to 20 μM \([γ-\text{ATP}]\)) but does result in increased phosphorylation of other proteins throughout the gel.

Effect of Mg\(^{2+}\) and Ca\(^{2+}\) on Phosphorylation—The concentration of Mg\(^{2+}\) required for half-maximal cyclic AMP-dependent phosphorylation is about 0.2 mM (Fig. 6, inset). The optimal concentration of Mg\(^{2+}\) for the cyclic AMP-dependent reaction is about 1 mM. The cyclic AMP-independent phosphorylation increases drastically as the concentration of Mg\(^{2+}\) is increased to 5 mM. However, cyclic AMP-stimulated phosphorylation is slightly decreased at these high concentrations of Mg\(^{2+}\). The ratio of cyclic AMP-dependent to cyclic AMP-independent phosphorylation is decreased from 1 to 0.2 as Mg\(^{2+}\) is increased from 1 to 5 mM. Disc gel electrophoresis reveals that at 5 mM Mg\(^{2+}\) the cyclic AMP-dependent incorporation of \(^{32}P\) into the 22,000 and 16,000 molecular weight peaks is slightly decreased concomitant with a substantial increase in the background radioactivity present throughout the gel. As with lower concentrations of Mg\(^{2+}\), no radioactivity is detected in Peaks I or II with 5 mM Mg\(^{2+}\) if cyclic AMP is absent in the incubation mixture.

Under the standard conditions of assay, Ca\(^{2+}\) effectively inhibits the endogenous phosphorylation of fat cell membranes (Fig. 7). The concentration of Ca\(^{2+}\) required for half-maximal inhibition of the cyclic AMP-dependent phosphorylation is about 0.1 mM; 1 mM Ca\(^{2+}\) nearly completely inhibits the cyclic AMP-dependent phosphorylation. Cyclic AMP-independent phosphorylation is also decreased by adding Ca\(^{2+}\).

Characterization of \[^{32}P\]Phosphate Incorporated into Membranes—Enzymatic digestions indicate that the phosphorylated membrane components are of protein composition (Table I). Incubation of phosphorylated membranes (which have been...
Phosphorylated membranes (33 μg in 0.1 ml), prepared as described under "Materials and Methods" with [γ-32P]ATP (10 μM, 2 × 10^6 cpm) and 1.5 μM cyclic AMP, were incubated for 30 min at 37° in 100 mM Tris-HCl (pH 7.0) with the indicated enzyme (1 mg per ml). Some incubation mixtures also contained 20 mM MgSO_4, 10 mM (staphylococcal nuclease) or 20 mM (phospholipase C) CaCl_2. Samples (0.1 ml) were incubated at 37° for 30 min in 100 mM sodium acetate buffer (pH 5.8) with 1 mM hydroxylamine. All incubations were stopped by the addition of equal volumes of 30% trichloroacetic acid containing 0.1 M phosphoric acid. The samples were centrifuged and the radioactivity in the pellets was measured by liquid scintillation counting. Extraction of lipids was carried out with 1-ml mixtures of ethanol-ether (1:1) and chloroform-methanol-HCl (20:10:0.1) at 37°; the organic phases were separated by aspiration, and the radioactivity remaining in aqueous phase was measured.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>32P incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Cyclic AMP (+)</td>
</tr>
<tr>
<td>None</td>
<td>1370 ± 50</td>
</tr>
<tr>
<td>Pronase</td>
<td>480 ± 40</td>
</tr>
<tr>
<td>Trypsin</td>
<td>520 ± 40</td>
</tr>
<tr>
<td>Nuclease</td>
<td>1220 ± 30</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>1220 ± 30</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1210 ± 30</td>
</tr>
<tr>
<td>Ethanol-ether</td>
<td>1250 ± 20</td>
</tr>
<tr>
<td>Chloroform-methanol-HCl</td>
<td>980 ± 10</td>
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</tbody>
</table>

* Expressed as counts per min ± S.E.

Washed extensively with trichloroacetic acid and NaOH followed by extensive washing with neutral buffers) with either staphylococcal nuclease or phospholipase C does not remove 32P phosphate from the membrane fraction. However, digestion with pronase or trypsin readily releases the 32P radioactivity from the membrane into a form which is trichloroacetic acid-soluble (Table 1).

Incubation of the 32P-phosphorylated membranes with 1 mM hydroxylamine at pH 5.5 does not release the incorporated 32P-phosphate (Table 1), indicating that the phosphate is not associated with membrane proteins through labile acyl phosphate bonds and suggesting that the linkage to membrane proteins may be through phosphoryl esters. Extraction of the membranes with the organic solvent, ethanol-ether (1:1), does not remove significant quantities of 32P (Table 1). However, extraction with chloroform-methanol-HCl (20:10:0.1) removes 50% of the radioactivity, indicating that at least some of the phosphoproteins may be lipophilic or hydrophilic in nature.

Well washed samples of 32P-labeled membrane proteins were hydrolyzed in 6 N HCl at 105° for 4 hours and subjected to high voltage electrophoresis using 32Porthophosphate, phosphoserine, and phosphothreonine as markers. At least 95% of the radioactivity (corrected for hydrolyzed 32Pphosphate) incorporated into the membranes as a result of cyclic AMP-dependent phosphorylation is present in the phosphoserine peak, and less than 5% is found in the phosphothreonine peak (Fig. 8). Cyclic AMP thus appears to stimulate the incorporation of 32Pphosphate nearly exclusively into protein-bound phosphoserine.

In separate experiments membranes were partially hydrolyzed by heating at 105° in 2 N HCl for 4 hours. Electrophoresis reveals that substantial amounts of radioactivity remain at the origin, that very little 32Pphosphothreonine is detectable, and that most of the radioactivity migrates as 32Pphosphoserine. The cyclic AMP-enhanced phosphorylation is reflected by increased radioactivity in both the origin and the phosphoserine peaks.

**Effect of Varying Concentration of Cyclic AMP and Cyclic GMP on Phosphorylation**—The concentration of cyclic AMP required to produce half-maximal effects on 32P incorporation is about 0.4 μM (Fig. 9). Maximal effects occur with 1.5 μM cyclic AMP, and concentrations higher than 10^-5 M result in a relative inhibition of the cyclic AMP effect. Cyclic AMP at 0.2 mM decreases the amount of 32P incorporated to the level measured in the absence of this cyclic nucleotide. Cyclic GMP at concentrations ranging from 10^-7 to 10^-5 M do not decrease significantly the amount of 32P incorporation observed with 1.5 μM cyclic AMP (Fig. 9). Cyclic GMP alone slightly stimulates 32P incorporation at concentrations of 0.1 to 0.5 μM (Fig. 10). Cyclic GMP at 0.1 mM stimulates 32P incorporation significantly, and disc gel electrophoresis reveals that the same protein peaks are labeled as are observed with cyclic AMP.

**Effect of Phosphonium Anologs of ATP, Nucleotide Triphosphates, and Phloretin**—We have recently reported that the ATP-dependent inhibition of insulin-stimulated glucose transport in intact rat fat cells, as well as the incorporation of 32P from [γ-32P]ATP into two specific membrane components, are protected by App(CH_2)pp and by phloretin (47). Fig. 3D shows that phloretin (1 mM) decreases substantially the cyclic AMP-stimulated phosphorylation of the two specific proteins in purified membranes. [γ-32P]ATP incorporation into the fat cell membrane protein is decreased by 1 mM phloretin (Fig. 9) and 1 mM App(CH_2)pp (Fig. 10) at all concentrations of cyclic AMP. GTP and ITP (1 mM) do not affect phosphorylation under the standard conditions of assay.

**Studies with Fat Cell Membranes from Obese Rats and Guinea Pigs**—Cyclic AMP-dependent incorporation of 32Pphosphate from [γ-32P]ATP into obese rat (300 to 600 g) purified membranes is less than that of the normal (about 150 g) rat membranes

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*Fig. 8. High voltage electrophoresis of a limited acid hydrolysate of fat cell membranes phosphorylated in the absence ( ) and presence ( ) of 1.5 μM cyclic AMP. The well drained, phosphorylated fat cell membranes were hydrolyzed in 1 ml of 6 N NaOH at 105° for 4 hours in tubes sealed under vacuum. The hydrolysate was subjected to electrophoresis on Whatman No. 3MM paper for 1 hour at 2500 volts and at pH 1.9 (2.5% formic acid, 7.8% acetic acid). [γ-32P]Phosphate, phosphoserine, and phosphothreonine were used as markers.*
Effect of varying the concentration of cyclic AMP (○) and cyclic GMP (●) on the endogenous phosphorylation of fat cell membranes in the presence of 1.5 μM cyclic AMP. The effect of phloretin on the membrane protein phosphorylation in the absence and presence of various concentrations of cyclic AMP (0) is also shown. The incubation conditions were as described in Fig. 1.

**TABLE II**

Endogenous phosphorylation of fat cell membranes from obese rats
and guinea pigs

The preparation of fat cell membrane fractions and the incubation (1 min) conditions were as described under "Materials and Methods" and Fig. 1 except for the variation in cyclic AMP concentration.

<table>
<thead>
<tr>
<th>Source of membrane</th>
<th>[32P] incorporated at cyclic AMP concentration:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Rat (120-140 g)</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Rat (500-600 g)</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Guinea pig (200-300 g)</td>
<td>9.4 ± 0.1</td>
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</table>

* Expressed as picomoles of [32P] per mg of membrane protein ± S.E.
+ Tests of paired analysis show the value is significantly different from the control (0 cyclic AMP) at the level of p < 0.01.
+ Significance is p > 0.05.

when expressed on the basis of membrane protein (Table II). Under the same conditions, 10⁶ cells from normal control rats yield 1.3 mg of membrane protein while the yield is 3.4 mg with cells from obese rats. Since the amount of membrane protein per cell thus appears to be higher in obese rats, the [32P] incorporation is not less in these cells if it is expressed per cell number. Disc gel electrophoresis reveals very similar if not identical [32P] phosphate patterns with membranes from normal and obese rats. Thus, no abnormality in the endogenous membrane kinase or its substrates can be detected which may be related to the insulin resistance (60-62) of the obese fat cell. This is consistent with the ability of exogenous ATP to inhibit insulin-stimulated n-glucose transport in these cells (data not shown).

Although in rat fat cells glucose transport appears to be the rate-limiting process in the over-all process of glucose oxidation when the concentration of glucose is 0.2 mM or lower (47, 64), it is not known if the same is true in guinea pig fat cells. Lowering the concentration of glucose from 6.2 mM to 20 μM only results in a very slight improvement of the response of guinea pig fat cells to insulin (Table III); the rates of glucose oxidation are increased by 45% and 110% using 0.2 mM and 20 μM glucose,
**TABLE III**

**Different effects of insulin and ATP on glucose oxidation in epididymal adipose fat cells isolated from guinea pigs and rats**

Fat cells (about 10^7 cells) from guinea pigs or rats were incubated for 14½ hours at 37° in 1.25 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (w/v) albumin and 20 μM or 0.2 mM 3-O-methyl-14C-glucose (5 μCi per μmole) in the absence of insulin and ATP as indicated.

![Table III](image)

**TABLE IV**

**Insulin stimulation of uptake of 3-O-methyl-D-glucose in fat cells from rats and guinea pigs**

Fat cells from rats or guinea pigs were incubated at 24° with 120 microunits of insulin per ml for 30 min, or with 0.1 mM phloretin for 10 min in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% (w/v) albumin. Uptake was initiated by the addition of 20 μM 3-O-methyl-D-[methyl-3H]glucose (specific activity, 1.2 Ci per μmole). After incubating at 24° for 15 and 60 s, 0.1 ml of the cell suspension (about 1.3 × 10^6 cells) was transferred to a microfuge tube containing 0.2 ml of silicone fluid and centrifuged for 15 s with a Beckman microfuge 152 (47, 65). The cells, which remain on the top of the oil, are thus quickly separated from incubation medium since this traverses through the layer of silicone fluid to the bottom of the tube. The cells 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 in the presence of a scintillation fluor consisting of 10 ml of TLX toluene fluorocarbonyl (Beckman) and 2 ml of Bio-Solv solubilizer BBS-3 (Beckman).

![Table IV](image)

* Counts per min of 14CO_2 produced in 1½ hours; average ± S.E.

* Twenty-four units per mg.

respectively. In rat fat cells the enhancement due to insulin is 350% and 500% for 20 μM and 0.2 mM glucose, respectively.

The resistance of glucose transport to insulin in the cells of guinea pigs was confirmed by direct measurements of the rate of uptake (47) of the nonmetabolized sugar, 3-O-methyl-D-[methyl-3H]glucose (Table IV). Whereas in rat fat cells the uptake of 3-O-methyl-D-glucose at 15 s in the presence of insulin (120 microunits per ml) is greater than that seen at 60 s in the absence of insulin, only a very slight effect of insulin is observed during the entire time course with the guinea pig fat cells. Furthermore, it is especially pertinent that the insulin resistance of guinea pig fat cells appears to be selectively restricted to the process of glucose transport since excellent antilipolytic effects of insulin are obtained with these cells (Table V).

As described earlier (47), low concentrations of ATP added exogenously suppress the insulin-stimulated but not the basal rates of glucose transport in rat fat cells. In contrast, the residual, small effect of insulin on glucose oxidation in guinea pig fat cells is not suppressed at all by exogenous ATP (Table III).

**DISCUSSION**

We have previously reported (47) that exogenous ATP, when applied to rat fat cells at low concentrations (10^-3 M), can quickly, selectively, and irreversibly inhibit insulin-stimulated glucose transport. After incubating the intact cells with [γ-32P]ATP, the plasma membrane-rich subcellular fraction was
TABLE V

Insulin sensitivity of rat and guinea pig fat cells to lipolytic response induced by (-)-norepinephrine

Fat cells were incubated at 37°C for 2 hours in Krebs-Ringer-bicarbonate buffer containing 3% albumin and, where indicated, (-)-norepinephrine and insulin. Glycerol was determined by the method of Ryley (66).

<table>
<thead>
<tr>
<th>Cells</th>
<th>(-)-Norepinephrine</th>
<th>Insulin*</th>
<th>Glycerol released in 2 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/ml</td>
<td>microunits/ml</td>
<td>μmoles/m mole triglyceride</td>
</tr>
<tr>
<td>Rat fat cells</td>
<td>None</td>
<td>None</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>None</td>
<td>42.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>500</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>Guinea pig cells</td>
<td>None</td>
<td>None</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>None</td>
<td>18.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>500</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

* Twenty-four units per mg.

Fig. 12. Sodium dodecyl sulfate disc gel electrophoretic patterns obtained from partially purified guinea pig fat cell membranes phosphorylated for 1 min in the absence (○) and presence (●) of 1.5 μM cyclic AMP. Details as in Fig. 3.

Fig. 13. Comparison of sodium dodecyl sulfate disc gel electrophoretic patterns prepared from normal rat and guinea pig purified fat cell plasma membranes.

purified from these cells contained two specifically phosphorylated components having apparent molecular weights of about 22,000 and 16,000 as determined by disc gel electrophoresis (47). The phosphorylation of these two proteins was prevented by phloretin and by App(CH₃)p, and the phosphorylation correlated well with the inhibition of insulin-stimulated glucose transport. It was suggested that these two membrane proteins might be related to insulin-sensitive glucose "carriers," and that the state of phosphorylation of these components may be at least one mechanism by which the effects of insulin on glucose transport can be mediated.

In the present studies, purified fat cell plasma membranes are shown to possess an endogenous protein kinase which can phosphorylate endogenous membrane proteins. In the absence of cyclic AMP, no specific proteins are selectively phosphorylated. However, in the presence of low concentrations (1 μM) of this cyclic nucleotide, two specific proteins with apparent molecular weights of 22,000 and 16,000 become phosphorylated. It is not possible at this time to ascribe the cyclic AMP-dependent phosphorylation reaction to stimulation of the activity of a protein kinase enzyme. The fact that phosphorylation is virtually complete in a few minutes while the degree of phosphorylation is maintained for more than 30 min, even when the [γ-³²P]ATP in the medium is nearly exhausted, suggests that specific phosphatases, if present, are relatively inactive under these conditions. However, it is not possible to exclude the possibility that cyclic AMP acts by inhibiting the activity of a specific protein phosphatase.

As observed in intact cells (47), the cyclic AMP-dependent phosphorylation of fat cell membranes is inhibited by the competitive antagonist of α-glucose transport, phloretin, as well as by the ATP analog, App(CH₃)p. The cyclic AMP-dependent reaction occurs very quickly, being essentially complete within 1 min, even at 24°C. Studies of enzymatic digestions indicate that phosphorylation occurs on proteins, although the partial extractability of radioactivity with certain organic solvents suggests that these proteins may have hydrophobic properties. Limited acid hydrolysis of the phosphorylated membranes followed by high voltage electrophoresis indicates that over 95% of the ³²P is present as phosphoserine.

The relatively low molecular weight of the two specifically phosphorylated membrane components is in contrast to the relatively high molecular weight of endogenously phosphorylated membrane proteins which have been described in other tissues. In synaptic membranes two cyclic AMP-dependent proteins with molecular weights of 86,000 and 48,000 are phosphorylated by endogenous kinases (7). The cyclic AMP-dependent endogenous phosphorylation of human erythrocyte ghosts involves two proteins with molecular weights of about 22,000 and 16,000.

It should be noted that in other studies (7, 11, 12, 24) of different membrane phosphoproteins the possible effect of dithiothreitol on molecular weight was not reported, and it is not always clear whether the membranes were heated in the presence of sodium dodecyl sulfate before gel electrophoresis.
80,000 and 50,000 (11), or proteins with a molecular weight of greater than 90,000 (12). Andrew et al. (23), on the other hand, have recently described the endogenous phosphorylation of three specific rat skeletal muscle membrane proteins whose molecular weight was less than 30,000. Cyclic AMP, however, was essentially without effect on the phosphorylation of these muscle membrane proteins. Nevertheless, because of the insulin sensitivity of muscle with respect to glucose transport, it is interesting to speculate on the possible identity of these membrane proteins with those described for fat cell membranes in the present studies.

The two specifically phosphorylated proteins in the fat cell membranes represent only a small fraction of the total membrane protein. The radioactive peaks observed on disc gel electrophoresis do not correspond to protein-staining bands; it can be calculated from the $^{32}$P incorporated (about 2 pmol per mg of protein) and from an assumed molecular weight of about 20,000 that each radioactive peak represents not more than 50 ng of protein. Detection of this phosphorylation reaction requires the use of $[^{32}P]ATP$ of very high specific activity (10 to 50 Ci per mmole). By labeling the intact fat cells (before preparation of the membranes) with the specific plasma membrane markers, $^{125}$I-labeled wheat germ agglutinin and $^{35}$S-labeled insulin, as described under "Materials and Methods," it is possible to estimate accurately the yield (usually near 40%) of membrane protein obtained from a known number of intact cells. It is thus possible to calculate that the total number of sites which can be phosphorylated in the purified membranes corresponds to a number that probably does not exceed 50,000 per cell. This is a very small number, especially when the relatively large size (diameter about 30 to 50 μm) of the cells is considered. The number of insulin and glucagon receptors per fat cell has been estimated to be in the same range.

The relatively small quantity of phosphorylated protein, the rapidity with which these proteins are phosphorylated, the endogenous membrane location of these proteins as well as of the enzymes involved in their phosphorylation, the highly specific nature of the proteins which are phosphorylated, and the absolute dependence of the specific phosphorylation reaction on the presence of cyclic AMP all suggest that the processes of phosphorylation and dephosphorylation of these two proteins may be related to important regulatory functions at the level of the cell membrane in the intact cell. The fact that the same two proteins can be specifically phosphorylated by brief treatment of intact cells with low concentrations of $[^{32}P]ATP$ (47), that this phosphorylation in the intact cells can be correlated with the specific suppression of the insulin-stimulated but not the basal rates of D-glucose transport (47), and that the effects of ATP on glucose transport as well as on phosphorylation either in intact cells or in purified membranes can be inhibited by phloretin and by App(CH₂)p suggest that the phosphorylation reaction under study may be related to the processes by which glucose transport is regulated by insulin. The possible participation of membrane phosphorylation in the regulation of membrane permeability has also been implicated in brain (7) and toad bladder (18, 22).

The specific membrane phosphorylation reaction described here may thus serve as a mechanism by which insulin regulates glucose (and perhaps other) transport through changes in the local concentration of cyclic AMP and, perhaps, cyclic GMP. Although insulin can decrease the total intracellular levels of cyclic AMP under certain circumstances (25-30), it has been very difficult to relate various effects of insulin to changes in the over-all cell levels of this cyclic nucleotide (67-70). However, physiological concentrations of insulin can directly inhibit the basal or stimulated activity of adenylate cyclase in isolated membrane preparations (31-37), and treatment of intact cells with insulin also causes an increase in the activity of a membrane-bound cyclic AMP phosphodiesterase which can be measured upon disruption of the treated cells (38-40). Since the substrates and enzymes involved in phosphorylation are present entirely in the cell membrane, it may be inappropriate to expect that the effective concentrations of cyclic AMP in the cell membrane, or more specifically in the vicinity of the specific membrane kinase involved, should be related to the total intracellular levels of the cyclic nucleotide. Thus, attempts to reconcile total cyclic AMP levels with the behavior of a cyclic AMP-sensitive process in the cell membrane may give misleading results. The actual activity of the membrane-bound enzymes catalyzing the synthesis and breakdown of the cyclic nucleotide, or the accessibility of the membrane kinase to cyclic AMP, ATP, or the protein substrate, may more accurately reflect the possible regulatory state of the system.

It is pertinent, for example, that in intact fat cells phosphorylation and inactivation of insulin-stimulated glucose transport occur very rapidly with exogenous concentrations of ATP which are at least two orders of magnitude lower than the intracellular "levels" of this nucleoside triphosphate (47). This has led to the speculation that an important factor in the regulation of this phosphorylation reaction may be the accessibility of ATP to the specific membrane-bound enzymes involved in this reaction. Similarly, although in the purified membranes specific membrane phosphorylation is absolutely dependent on the presence of cyclic AMP, phosphorylation of these same proteins in the intact cell does not require and is not modified by the addition of exogenous cyclic AMP (47). Thus, as with measurements of total levels of cell constituents, the metabolic effects of exogenously added substances must be interpreted cautiously, especially when membrane-localized functions are being studied.

The effects of exogenous cyclic AMP and its analogs on fat cell glucose oxidation have resulted in conflicting and paradoxical results that are difficult to interpret (42-46). The well known ability of hormones which are believed to act by stimulating the activity of adenylate cyclase to enhance (weakly) glucose oxidation in fat cells may appear to contradict the view that a fall in cyclic AMP may somehow be related to stimulation of glucose transport and oxidation. It can be demonstrated, however, that the effects of hormones such as adrenocorticotropin, l-norepinephrine, and glucagon on oxidation under conditions where glucose transport is rate-limiting (i.e. low glucose concentrations) are extremely weak (Table VI). Furthermore, these hormones markedly decrease the insulin-stimulated rates of glucose oxidation (Table VI), as expected if the glucose transport mechanisms are inversely related to the state of activity of the membrane-bound adenylate cyclase. These results are in accord with studies which indicate that the small stimulatory effects of such hormones on glucose oxidation are mediated by mechanisms different from those involved with insulin (76-77). It is also notable that cholera toxin, a protein that irreversibly stimulates the activity of adenylate cyclase, inhibits profoundly the ability of insulin to stimulate glucose oxidation in fat cells (Table VI). The inhibition of the insulin effect by cholera toxin demonstrates a lag period of about 60 min, which corre-
sponds to the latency observed for the stimulation of adenylyl cyclase by this protein.4

It has recently been described (41) that insulin causes a rapid elevation of the intracellular levels of cyclic GMP in isolated fat cells. Although at present there is no evidence to indicate that this cyclic nucleotide is involved in the regulation of glucose transport or in the membrane phosphorylation described in this report, future investigations must consider the possible role of cyclic GMP.

The endogenous phosphorylation of plasma membranes from guinea pig fat cells is unaffected by cyclic AMP and does not show phosphorylation of the low molecular weight proteins observed in fat cell membranes. Guinea pig fat cells are resistant to insulin with respect to glucose transport but not lipolysis. In the membranes from guinea pig cells, phosphorylation occurs predominantly in a protein having a molecular weight of about 50,000. Also, the small effect of insulin on glucose oxidation is not affected by exogenous ATP, in contrast to the effects observed with rat fat cells (Table III). The possibility must be considered that the selective resistance of glucose transport to insulin in this species may be related to an alteration in the membrane phosphorylation system. For example, this could occur if the specific membrane proteins (kinase or substrates) of this system were relatively deficient or absent, or if the specific low molecular weight proteins were already fully phosphorylated. In the obese rat insulin resistance cannot be explained by similar considerations since the cyclic AMP-dependent phosphorylation is quite intact in membrane preparations obtained from these cells. This does not, however, exclude the possibility that in the intact obese fat cell there may be an impairment in the integrated function of this phosphorylating system.

The possibility must be considered (47) that the membrane phosphorylation system described here may be involved in the long term regulation of insulin sensitivity to glucose transport. The ability of exogenous ATP to cause phosphorylation and to profoundly suppress the insulin response in the absence of added cyclic AMP may suggest the possibility that regulation of phosphorylation may be subject to control by mechanisms other than the availability of cyclic AMP.5 Furthermore, the relatively protracted biological effects of phosphorylation in the intact cell (47) suggest that this system may be involved in long term regulatory mechanisms. The possible involvement of this system in the mechanism of insulin-stimulated glucose transport, and in regulating insulin sensitivity in conditions characterized by insulin resistance, remains to be elucidated.

REFERENCES


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4 Attempts to obtain direct hormonal effects (insulin, epinephrine) in the phosphorylation assay have not been successful, and incubation of intact cells with these hormones prior to the purification of membranes has had no consistent effect.

---

TABLE VI

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conversion of [14C]Glucose to [14CO2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15,200 ± 800</td>
</tr>
<tr>
<td>Adrenocorticotropicin, 10⁻⁶ M</td>
<td>17,100 ± 600</td>
</tr>
<tr>
<td>Glucagon, 2 x 10⁻⁷ M</td>
<td>15,900 ± 300</td>
</tr>
<tr>
<td>(-)-Norepinephrine, 10⁻⁴ M</td>
<td>19,100 ± 600</td>
</tr>
<tr>
<td>Insulin, 1.4 x 10⁻⁸ M</td>
<td>54,700 ± 1,300</td>
</tr>
<tr>
<td>Insulin, 1.4 x 10⁻⁶ M</td>
<td>35,500 ± 400</td>
</tr>
<tr>
<td>+ Glucagon, 2 x 10⁻⁷ M</td>
<td>33,700 ± 800</td>
</tr>
<tr>
<td>+ (-)-Norepinephrine, 10⁻⁴ M</td>
<td>38,800 ± 1,200</td>
</tr>
<tr>
<td>None</td>
<td>6,800 ± 200</td>
</tr>
<tr>
<td>Cholera toxin, 10⁻⁴ M</td>
<td>7,200 ± 300</td>
</tr>
<tr>
<td>Insulin, 0 x 10⁻¹¹ M</td>
<td>44,900 ± 800</td>
</tr>
<tr>
<td>Insulin, 6 x 10⁻¹¹ M</td>
<td>7,900 ± 600</td>
</tr>
</tbody>
</table>

* Counts per min of [14CO2] produced in 1 hour; average value ± S.E.
Cyclic Adenosine Monophosphate-dependent Phosphorylation of Specific Fat Cell Membrane Proteins by an Endogenous Membrane-bound Protein Kinase: POSSIBLE INVOLVEMENT IN THE REGULATION OF INSULIN-STIMULATED GLUCOSE TRANSPORT
Kwen-Jen Chang, Norman A. Marcus and Pedro Cuatrecasas


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