Insulin Decreases Phosphoenolpyruvate Carboxykinase (GTP) mRNA Activity by a Receptor-mediated Process*

(Received for publication, August 24, 1981)

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The mRNA that codes for phosphoenolpyruvate carboxykinase accounts for approximately 0.2% of the protein synthesized in H4IIEC3 hepatoma cells maintained for 24 h in serum-free medium containing N6,02'-dibutyryl CAMP and theophylline. This value decreases to 0.04% within 3 h after the addition of insulin. Maximal effects are produced by 10^-10 m insulin, and half-maximal deinduction of both the relative rate of synthesis of P-enolpyruvate carboxykinase and mRNA coding for P-enolpyruvate carboxykinase activity occurs at approximately 2 x 10^-11 m insulin. Porcine proinsulin is 4% as potent as porcine insulin since half-maximal deinduction of mRNA coding for P-enolpyruvate carboxykinase occurs at 5 x 10^-11 m. The concentration of proinsulin required to inhibit 125I-insulin binding by 50% is 2 x 10^-7 m, as compared to 6 x 10^-7 m for insulin; thus, the decreased sensitivity of this deinduction to proinsulin parallels the decreased binding affinity H4IIEC3 cells have for proinsulin as compared to insulin. These data indicate that insulin regulates P-enolpyruvate carboxykinase synthesis through a receptor-mediated process, that the effect occurs when less than 2% of the insulin receptors are occupied, and that this effect is exerted prior to the level of mRNA translation.

Several important questions remain to be answered about how insulin regulates the synthesis of specific proteins. A principal question is whether insulin itself, as opposed to hormones or metabolites released in response to insulin, is responsible for the regulation of the many proteins alleged to be affected by this hormone. A second question concerns whether the effects are mediated through the insulin receptor. Whereas the effects of insulin on glucose transport and oxidation seem to be initiated by, and tightly coupled to, the interaction of insulin with its specific receptor and synthesis of a specific protein have not been reported.

The use of cell culture, in which the environment can be measured and controlled, combined with the newer techniques of molecular biology, allows such questions to be asked. In H4IIEC3 cells, a permanent tissue culture cell line derived from a rat hepatoma (9), the rate of synthesis of the enzyme P-enolpyruvate carboxykinase, a critical, rate-limiting enzyme in the gluconeogenic pathway, is increased by glucocorticoids and cyclic AMP and decreased by insulin (10, 11) just as in liver. Using this cell line, we are able to separate insulin effects from those of other hormones and can show that physiological concentrations of insulin elicit the well defined biological response of suppression of P-enolpyruvate carboxykinase synthesis. This effect is due to a selective decrease in the activity of the mRNA that codes for this protein and appears to be mediated through the insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Swim's 77 medium was purchased from Grand Island Biological Corp.; serum was from Flow Laboratories, Inc. and BtZcAMP was from Sigma Chemical Co. Porcine insulin and porcine proinsulin were the generous gifts of Dr. Ronald Chance of Lilly Research Laboratories. [3H]Leucine (40-60 Ci/mmol) and reticulocyte lysate translation kits with [35S]methionine were obtained from New England Nuclear. Sodium ([125]Iodide (carrier-free) was purchased from Amersham. [125I]Insulin (150-200 Ci/g) was prepared by the chloramine-T procedure. The preparation and characterization of sheep antiserum directed against rat cytoplasmic P-enolpyruvate carboxykinase has been described before (12). Cowan Strain I of Staphylococcus aureus was obtained from American Type Culture Collection and was grown and prepared as described by Kessler (13).

**All other chemicals were reagent grade.**

**Cell Culture**—H4IIEC3 cells, obtained from Van Potter of the University of Wisconsin, were grown to confluency in T-150 flasks (Corning) in modified Swim's 77 (14) containing 2.5% newborn calf serum and 2.5% calf serum. The final concentration of glucose was 6.1 mM. The experiments were generally performed as follows. Twenty-four h prior to harvest, the cells were placed in medium lacking serum, with 0.5 mM BtZcAMP and 1.0 mM theophylline in all but "control" cultures. Three h prior to harvest, insulin was added to and leucine omitted from the medium. [3H]Leucine (1.7 μCi/ml) was added for the last 0.5 h, after which the cells were harvested and the in vivo synthesis of P-enolpyruvate carboxykinase and RNA extraction were performed as described below.

**In Vito Synthesis Measurements**—Cells were scraped into 2.5 ml of a buffer containing 0.25 M sucrose, 150 mM KCl, 1.0 mM EDTA, 10 mM imidazole, pH 7.4, 0.02% sodium azide. An aliquot containing 10^7 cells was sonicated at the lowest setting of a Branson Sonifier equipped with a microtip. Quantitation of radioactivity incorporated into P-enolpyruvate carboxykinase was accomplished by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; radioactivity incorporated into total protein was determined by trichloroacetic acid precipitation, as described by Besse.

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*This work was supported by United States Public Health Service Grants AM20858 and AM25285 (Diabetes and Endocrinology Research Center) and by Veterans Administration research funds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†† The abbreviations used are: BtZcAMP, N6,02'-dibutyryl cAMP; mRNA {poly(A)}+RNA, polyadenylated RNA.
et al. (12). Results are expressed as (radioactivity in P-enolpyruvate carboxykinase) (radioactivity in trichloroacetic acid-insoluble material) x 100.

Isolation of Poly(A)+RNA and In Vitro Translational Assay—RNA was extracted from 1.5 x 10^9 HJIEC3 cells essentially as described for HTC cells by Olson et al. (15), with the exception that only one passage over oligo(dT)-cellulose was done. Poly(A)+RNA was dissolved in 10 mM sodium chloride at a final concentration of 250–330 μg/ml and stored at −80 °C until use. The in vitro translational activity of mRNAPEPCK in poly(A)+RNA samples was determined in an 18.7-μl reticulocyte lysate assay as described by Beale et al. (12). mRNAPEPCK activity is expressed as a percentage of total mRNA activity (i.e., radioactivity in P-enolpyruvate carboxykinase/radioactivity in trichloroacetic acid precipitable material) x 100). The drawing of gel profiles and subsequent calculations were performed using computer with a Tektronix Graphics terminal.

Insulin Receptor Studies—Binding studies were performed using adherent, subconfluent monolayer cultures grown in modified Swim's 77 medium in T-25 flasks. The culture medium was aspirated from the flasks; then the cells were washed twice with 2 ml of 0.1 M (2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at pH 7.8. Cells were incubated with 200 pg of 125I-insulin and a range of concentrations of unlabeled insulin (10^-11 to 10^-5 M) in a final volume of 1.25 ml/flask. After 2.5 h at 22 °C, the incubation medium was removed and the cells were rapidly washed three times with 2 ml of cold buffer. Under these conditions, steady state binding was reached by 2 h and degradation of 125I-insulin was <10%, as determined by precipitation with 5% trichloroacetic acid. Cell counts were performed on randomly chosen areas of the monolayer cultures using a calibrated eyepiece; 3000–5000 cells were counted per flask. The cells were then removed with 1.0 ml of 0.5 M NaOH (complete lysis was validated by visual inspection) and washed twice with H2O. Cell-associated radioactivity was determined in a Beckman gamma counter at 76% efficiency. Nonspecific binding was defined as the 125I-labeled insulin bound in the presence of 10^-8 M unlabeled insulin and was subtracted from total binding to yield specific binding.

Table I

<table>
<thead>
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<th>Exper-</th>
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<td>ient No.</td>
<td>Condition</td>
<td>P-enolpyruvate carboxykinase synthesis</td>
<td>mRNAPEPCK activity</td>
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<tr>
<td>I</td>
<td>Control</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
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<td></td>
<td>Bt2cAMP</td>
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<td>0.26 ± 0.01</td>
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<td>0.04 ± 0.00</td>
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<td>0.07 ± 0.01</td>
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<td>0.14 ± 0.02</td>
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<td>Bt2cAMP + insulin</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>III</td>
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<td>Bt2cAMP + insulin</td>
<td>0.04 ± 0.00</td>
<td>0.03 ± 0.00</td>
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<td>Bt2cAMP + insulin, 10^-11 M</td>
<td>0.14 ± 0.01</td>
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<td>Bt2cAMP + insulin, 10^-10 M</td>
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<td>0.02 ± 0.00</td>
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<td>0.02 ± 0.00</td>
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<td>Bt2cAMP + insulin, 5 x 10^-8 M</td>
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<td>0.03 ± 0.00</td>
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The regulation of P-enolpyruvate carboxykinase synthesis in HJIEC3 cells is illustrated in Table I. In three experiments, dibutyryl cyclic AMP, in the presence of an inhibitor of phosphodiesterase, more than doubled the rate of synthesis of P-enolpyruvate carboxykinase (average of 0.11 to 0.26%) and caused a proportional increase (0.09 to 0.19%) in the activity of mRNAPEPCK measured in the same cells. In such cells, treated so as to be synthesizing P-enolpyruvate carboxykinase at a high rate, insulin was a potent and specific inhibitor. Five nm insulin, a concentration readily achieved in the portal circulation after feeding, caused a decrease in the rate of synthesis of P-enolpyruvate carboxykinase to or below control levels within 3 h in each experiment. The average decrease in the three experiments was from 0.26 to 0.04% of total protein. This was accompanied by a parallel diminution of mRNAPEPCK activity, with the average being a decline from 0.19 to 0.04% of total poly(A)+RNA activity.

The observation that 5 nm insulin markedly repressed P-enolpyruvate carboxykinase synthesis and mRNA activity led us to test the effects of even smaller concentrations of insulin. As little as 10^-12 M insulin reduced the rate of P-enolpyruvate carboxykinase synthesis from 0.34% of total protein to 0.16% and decreased mRNAPEPCK translational activity from 0.17% of total polyA+RNA to 0.10% (experiment IV, Table I). The

Fig. 1. Effect of insulin and proinsulin on mRNAPEPCK activity and 125I-insulin binding. Experimental conditions are described under “Experimental Procedures.” The effect of insulin and proinsulin on mRNAPEPCK activity is shown in A. Each point represents the mean ± S.E. of translation assays on three samples of poly(A)+RNA, each of which included cells from three flasks of three. The dotted line marked insulin was constructed from data appearing in Table I, Experiment IV. The absence of a bar indicates that the S.E. was smaller than the diameter of the symbol. The value for no insulin was from flasks treated with Bt2cAMP and theophylline only. Arrows designate the positions on the curves used to determine a half-maximal value. Binding of 125I-insulin in the presence of various concentrations of porcine insulin and porcine proinsulin is shown in B.
concentration, it is apparent that a maximal effect of insulin receptors are occupied by the hormone. The same effect is made more apparent by the representation illustrated on mRNAPEPCK activity is seen when less than 2% of the receptors are occupied by insulin or proinsulin. This observation that the half maximal concentration required for receptor binding, and that insulins of different biological potency show parallel changes in binding activity. H411EC3 cells are extremely sensitive to insulin as evidenced by the observation that the half maximal concentration required for the repression of P-enolpyruvate carboxykinase synthesis and mRNAPEPCK activity is about 2 × 10^{-12} M (Fig. 2, Table 1). Similarly, the half-maximal concentration required for tyrosine aminotransferase induction (16) and stimulation of glucose oxidation (17) in these cells is 10^{-11} M. Our studies show that insulin decreases mRNAPEPCK in H411EC3 cells at concentrations considerably below those required for maximal binding of insulin to these same cells, as detected by a radioreceptor assay (Fig. 1). This observation is important since at high concentrations insulin may bind to growth peptide receptors (24) and, thus, could mediate effects through this mechanism rather than by the insulin receptor. The low concentrations used in this study make it very unlikely that insulin is depressing mRNAPEPCK activity by acting through the growth peptide receptor.

A maximal biological effect occurs when less than 2% of the insulin receptors are occupied by the hormone (Fig. 2). This implies that there are spare receptors and resembles a similar observation with respect to the stimulation of glucose oxidation by insulin (25). The role of spare receptors in insulin action has been discussed in detail (26, 27). In general, insulin binds to its receptor 15-20 times more avidly than proinsulin, and it is proportionately more effective in eliciting biological responses. The repression of mRNAPEPCK activity as a function of the log concentration of insulin and proinsulin is shown in Fig. 1A. Half-maximal suppression by insulin was achieved at about 2 × 10^{-12} M whereas a similar effect of proinsulin required about 5 × 10^{-11} M. This 1.4 log difference in biological effectiveness correlates very well with the affinity of binding of each ligand for the insulin receptor, as illustrated in Fig. 1B. The concentration of insulin required to decrease maximal binding of 125I-porcine insulin to H411EC3 cells by 50% was 6 × 10^{-7} M for insulin and 2 × 10^{-10} M for proinsulin, a 1.5 log difference. These values for insulin binding in H411EC3 cells agree very well with studies reported earlier by other groups (18, 17).

In addition to demonstrating that the order of potency of insulin and proinsulin for binding and repression of mRNAPEPCK agree very well, this study suggests that both eliciting biological effects when only a small fraction of the total receptors are occupied by insulin or proinsulin. This observation is made more apparent by the representation illustrated in Fig. 2. When the percentages of receptor occupancy and biological activity are expressed as a function of media insulin concentration, it is apparent that a maximal effect of insulin on mRNAPEPCK activity is seen when less than 2% of the receptors are occupied by the hormone. The same effect occurs with proinsulin (data not shown).

**DISCUSSION**

Insulin appears to play an important role in regulating the synthesis of a number of proteins in a variety of different tissues (6-8, 10, 11, 18-22). It is not certain whether it does this by enhancing the rate of translation of a fixed amount of mRNA as has been postulated (3, 4) or, as more recent evidence suggests, by changing the activity and/or amount of that mRNA (3-8, this paper). In addition, several general aspects of this role of insulin are unresolved. These include whether insulin itself is the proximate regulator, whether the effects occur within the physiological range of insulin concentration, and whether the effect is mediated by the insulin receptor or a growth peptide receptor.

In this study, H411EC3 cells, a permanent cell line derived from a rat hepatoma (9), were used to address these questions. Insulin represses P-enolpyruvate carboxykinase activity in these cells by decreasing the rate of synthesis of this protein (10, 11). Our data suggest that this is due to a selective and proportionate decrease in the activity of the mRNA that codes for P-enolpyruvate carboxykinase (Table I and Fig. 1). Since these effects are obtained in cells maintained for 24 h in culture medium lacking serum, the possibility that other hormones are involved is excluded. The necessity of being able to separate insulin effects from those of other hormones which mimic or oppose the actions of insulin is always important but is particularly critical in the case of enzymes such as P-enolpyruvate carboxykinase, since, in addition to insulin, its synthesis is known to be regulated by cyclic AMP, glucocorticoids (10, 11), and glucose (23).

The current model of insulin action implies that an interaction with the insulin receptor at the cell surface initiates all the subsequent effects of the hormone. Accordingly, one should be able to correlate insulin binding with its effects on mRNAPEPCK. Such an association ideally requires that both processes be quantitated in the same cell system, that the biological effect occur at or below the concentration required for receptor binding, and that insulins of different biological potency show parallel changes in binding activity. H411EC3 cells are extremely sensitive to insulin as evidenced by the observation that the half maximal concentration required for the repression of P-enolpyruvate carboxykinase synthesis and mRNAPEPCK activity is about 2 × 10^{-12} M (Fig. 2, Table 1). Similarly, the half-maximal concentration required for tyrosine aminotransferase induction (16) and stimulation of glucose oxidation (17) in these cells is 10^{-11} M. Our studies show that insulin decreases mRNAPEPCK in H411EC3 cells at concentrations considerably below those required for maximal binding of insulin to these same cells, as detected by a radioreceptor assay (Fig. 1). This observation is important since at high concentrations insulin may bind to growth peptide receptors (24) and, thus, could mediate effects through this mechanism rather than by the insulin receptor. The low concentrations used in this study make it very unlikely that insulin is depressing mRNAPEPCK activity by acting through the growth peptide receptor.

A maximal biological effect occurs when less than 2% of the insulin receptors are occupied by the hormone (Fig. 2). This implies that there are spare receptors and resembles a similar observation with respect to the stimulation of glucose oxidation by insulin (25). The role of spare receptors in insulin action has been discussed in detail (26, 27). In general, insulin binds to its receptor 15-20 times more avidly than does proinsulin and is likewise proportionately more effective in eliciting biological responses (1). This expected relationship is seen in mRNAPEPCK repression, as illustrated in Fig. 1, A and B. Interestingly, proinsulin also exerts its maximal effect on mRNAPEPCK when only 2% of the insulin receptors is occupied, even though it takes 20 times more proinsulin to achieve this degree of receptor occupancy. Since insulin and proinsulin usually bind to the growth peptide receptors with nearly equal affinity (28), and since very different concentrations of insulin and proinsulin are required to depress mRNAPEPCK, the possibility that this effect is mediated through the growth peptide receptor is essentially excluded.

Until recently, inferences about insulin action were made from indirect studies including the use of various inhibitors of macromolecular synthesis. It now is possible to directly test various hypotheses of the mechanism of action of insulin, and,
in the past year, this hormone has been shown to change the activity and/or amount of the mRNAs that code for the liver proteins tyrosine aminotransferase (6), albumin (7), and P-enolpyruvate carboxykinase (5, this paper), and for pancreatic amylase (8). To this paper, however, all demonstrations of an effect of insulin on either the amount or activity of a specific mRNA have employed intact animals (5–8). A number of problems and limitations are inherent in such studies. Proteins regulated by insulin are often affected by a number of other hormones and metabolic substrates. Insulin administration causes profound metabolic effects and thus may result in changes in blood levels of hormones or factors which, in themselves, mimic or counteract insulin effects. This complication can, in part, be controlled by the use of adrenalectomized rats injected with glucose, as was done in the study on tyrosine aminotransferase mRNA (6). Just as it is difficult to sort out the precise effects of individual hormones in intact animals, it is also not possible to study how such hormones interact to influence a given metabolic process. Another difficulty in interpreting the intact animal studies comes from the fact that large doses of insulin have generally been used; hence, the physiological relevance of the response cannot be assessed, nor can any relationship to insulin receptor activity be made. Finally, insulin often must be given over a long period of time. For example, the albumin and amylase mRNA responses required 1–2 days of insulin treatment (7, 8); hence, it is difficult to exclude the possibility that, in these instances, this hormone is not the proximate regulator.

Keeping these potential difficulties in mind, the most direct data suggest that insulin changes the synthesis of four proteins in proportion to the mRNAs coding for them. It is reasonable to assume that these are associated events. Since specific cDNA probes were used to demonstrate the changes in albumin (7) and amylase mRNA (8), one can now hypothesize that insulin effects changes in protein synthesis by changing specific mRNA levels.

In conclusion, we have shown that insulin itself affects H4IEC3 cell P-enolpyruvate carboxykinase synthesis by rapidly lowering the activity of the mRNA that codes for this protein. This is achieved at insulin concentrations at the lower end of the physiological range, and the process appears to be mediated through the insulin receptor. The observation that insulin decreases the activity of mRNAPm which provides new direction for studies of the regulation of this important enzyme and contributes to the formulation of insulin action described above. The H4IEC3 cell system is well suited for studies of the individual and concerted actions of insulin, cAMP, glucocorticoids, and glucose on P-enolpyruvate carboxykinase. We have recently synthesized and cloned a DNA complementary to mRNAPm which should facilitate such future studies.

Acknowledgments—We thank Steve Koch and Mike Peacock for technical assistance, Mark Granner for technical assistance and for writing the computer programs, and Janet Adams for secretarial assistance.

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