Insulin-induced proteins in the toad urinary bladder

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Insulin increases active sodium transport by the toad urinary bladder within 15 min, an effect which persists for at least 20 h. In tissues pre-treated with inhibitors of transcription or translation, sodium transport briefly increases after insulin addition but returns to basal levels within 60–90 min. We have studied the effects of insulin on the incorporation of radioactive amino acids into mucosal cell proteins. Insulin had no detectable effect on the uptake of amino acids nor on their incorporation into total protein; however, using a dual label technique, we found that insulin increases the incorporation of amino acids into specific soluble and plasma membrane proteins of the granular mucosal cell. The time course and dose–response relationship of the induction of protein synthesis by insulin suggest that these proteins may play a role in the sustained elevation of sodium transport by insulin.

We have studied the effects of insulin on protein synthesis in the toad urinary bladder and examined the possible relationship between insulin-induced protein synthesis and sodium transport. Interest in this question was raised by the findings of Wiesmann *et al.* (1976, 1977) that, in the toad bladder, the long term effects of insulin on sodium transport, i.e. those observed after the first 1 h of stimulation, were sensitive to actinomycin D. This observation, which was contrary to earlier reports that inhibitors of protein and RNA synthesis did not affect the stimulation of sodium transport by insulin in this and other epithelial tissues (Crabbé, 1973; Cox & Singer, 1977), suggested that the long-term transport response could be related to protein synthesis. We have used the toad urinary bladder, a tissue which bears many similarities to the mammalian distal nephron, to examine in more detail (1) the effects of transcriptional and translational inhibitors on insulin-enhanced transport and (2) the effects of insulin on protein synthesis. We found that inhibitors of protein or RNA synthesis do not block the early effects of insulin on transport, whereas the long-term increase in transport (after the first 1 h of stimulation) is blocked by cycloheximide, puromycin and actinomycin D (Cobb & Scott, 1979). We have also found that physiological levels of insulin induce the synthesis of specific proteins in the

granular (G) cell of the toad bladder; the relationship of these effects on protein synthesis to those on transport remains to be elucidated.

Materials and methods

Pig insulin was generously provided by Dr. Ronald Chance, Eli Lilly and Co., Indianapolis, IN, U.S.A. Radioactively labelled amino acids were obtained from New England Nuclear: L-[4,5-³H]-leucine (40 Ci/mmol), L-[³⁵S]-methionine (500–630 Ci/mmol); α -amino[methyl-³H]isobutyric acid (20 Ci/mmol), ³H-labelled L-amino acid mixture (NET-250) and ¹⁴C-labelled L-amino acid mixture (NEC-445) and from The Radiochemical Centre, Amersham: [2(*n*)-³H]-methionine (TRK.401) (2–10 Ci/mmol), ³H-labelled L-amino acid mixture (TRK.440) and ¹⁴C-labelled L-amino acid mixture (CFB.25). NP-40 was purchased from Particle Data Labs (Rockford, IL, U.S.A.), ampholytes from LKB (Rockville, MD, U.S.A.), and Dextran T-70 and Ficoll from Pharmacia.

Measurement of sodium transport

Female toads (*Bufo marinus*) from the Dominican Republic were maintained unfed in the laboratory for at least 10 days prior to killing. Hemibladders, excised from doubly pithed animals that had been kept in 0.6% NaCl for the previous 3–7 days, were mounted on glass tubes and immersed in an aerated amphibian Ringer solution (Scott *et al.*, 1974). Cycloheximide (1–2 μ g/ml) was added to the external (serosal) bath as indicated, 90–180 min prior to

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Abbreviations used: G, granular; MR, mitochondria-rich; SDS, sodium dodecyl sulphate.

the addition of insulin. The short-circuit current, a measure of active sodium transport by the tissue (Leaf *et al.*, 1957), was measured by the method of Bentley (1960) for up to 24 h after the addition of hormone.

Isolation of mucosal cells

Suspensions of unfractionated mucosal cells (8×10^6 cells/animal) with 60–65% viability, as well as preparations enriched in MR and G mucosal cells, were isolated as described previously (Scott *et al.*, 1974). The recovery of MR and G cells was in proportion to their representation in the total epithelial cell population; 15% of the total cells applied to the gradients [$(3-7) \times 10^7/38$ ml tube] were recovered in the MR cell-enriched fraction and 50% in the granular cell-enriched fraction. The viability of these cells was 91%, determined by Trypan Blue exclusion, decreasing by only 5% after 3–4 h. Cell enrichments (Scott *et al.*, 1974) were determined by carbonic anhydrase activity, electron microscopic appearance and size distribution of cells in each fraction.

Uptake and incorporation of radiolabelled amino acids into total protein

Intact bladders. Paired hemibladders were mounted on Luer-lock syringes and placed in one of two baths, both containing either ^{14}C -labelled amino acids ($1-5 \mu\text{Ci/ml}$) or amino [^3H]isobutyrate ($2 \mu\text{Ci/ml}$), one with and one without insulin. At various intervals bladders were removed from each bath and epithelial cells were removed by scraping and treated with 0.5 ml of 10% (w/v) trichloroacetic acid.

Isolated cells. MR and G cells from 10–20 toads were resuspended in 5–30 ml of Ringer's solution to which one of the following was added: $5-20 \mu\text{Ci}$ of L-[4,5- ^3H]leucine/ml, $5-20 \mu\text{Ci}$ of L-[^{35}S]methionine/ml, $5-10 \mu\text{Ci}$ of ^3H -labelled L-amino acid mixture/ml or $5 \mu\text{Ci}$ of ^{14}C -labelled L-amino acid mixture/ml. The cell suspensions were each divided into two equal parts, to one of which diluent was added and to one of which insulin ($10 \mu\text{U}-100 \text{mU/ml}$) was added. To measure the effect of hormone concentration on uptake of amino acids, the cell suspensions were each divided into five to ten aliquots to which various concentrations of insulin were added. After incubation, cells were treated with 10% (w/v) trichloroacetic acid. Each experiment was performed a minimum of three times.

Trichloroacetic acid-precipitated material was dissolved in 1 M-NaOH for measurement of protein (Lowry *et al.*, 1951). Beckman Tissue Solubilizer (0.5 ml) and a toluene-based scintillant (10 ml) were added to aliquots of supernatants and precipitates. Samples were analysed in a Beckman LS-9000 spectrometer with efficiencies of 43–45% and 82–85% for ^3H and ^{14}C or ^{35}S , respectively.

Dual isotope labelling of mucosal cell proteins

Intact tissues. Paired hemibladders were dissected from 60–80 toads and mounted as described. Insulin and a mixture of amino acids labelled with either ^3H ($5 \mu\text{Ci/ml}$) or ^{14}C ($5 \mu\text{Ci/ml}$), were added to the serosal bath of one set of bladders. A complementary mixture of either ^{14}C - or ^3H -labelled amino acids were added to the paired, control tissues. Following an incubation of either 1 or 3 h, the mucosal cells were recovered from both sets of tissues and combined, the MR and G cells were separated, the cells were disrupted and subjected to the subcellular fractionation procedures described below.

Isolated cells. The MR and G mucosal cells derived from 50–60 animals were suspended in 5–20 ml of Ringer's solution and each was divided into two parts. Insulin plus [^3H]methionine, [^{35}S]methionine, a ^3H -labelled L-amino acid or a ^{14}C -labelled L-amino acid mixture (all $20-50 \mu\text{Ci/ml}$) was added to one set and diluent plus the complementary, labelled precursor was added to the other. In some experiments one group of cells was preincubated with cycloheximide ($1 \mu\text{g/ml}$) for 1 h and then both groups were treated with insulin (100mU/ml). Following an incubation of 1, 2 or 3 h, the epithelial cells were sedimented, rinsed and the insulin-treated and control MR cells were each pooled, as were the G cells, and subcellular fractions were prepared.

Subcellular fractionation

Subcellular fractions of toad bladder epithelial cells were prepared using the procedure of Scott *et al.* (1979). The specific activity of 5'-nucleotidase in the plasma membrane fraction was enriched 11.3-fold over the homogenate, whereas specific activities of cytochrome oxidase and glucose-6-phosphatase in this fraction were 0.20- and 0.74-fold of the homogenate, respectively (Scott *et al.*, 1979). The ^{125}I specific activity of the radio-iodinated plasma membranes was 20-fold greater than that of the 5000 g pellet and 5-fold greater than that of the 90 000 g microsomal pellet.

Analysis of radioactively labelled proteins

SDS/polyacrylamide-gel electrophoresis. The membrane pellets were suspended in 50 mM-Tris/HCl (pH 6.8)/2 mM-EDTA/10% (w/v) sucrose, sonicated at 0°C and brought to final concentrations of 2% (w/v) SDS and 2 mM-dithioerythritol, as were the soluble fractions. The samples were heated to 100°C for 90 s and electrophoresed on SDS/polyacrylamide (8%) gels (Zahler, 1974). Gels were fixed and stained in 1% Amido Black in water/methanol/acetic acid (46:45:9, by vol.). The gels were destained by diffusion in acetic acid/water (7:93, v/v) and scanned in a Gilford Model 2320 gel

scanner prior to slicing. The slices (1.1 mm) were immersed in 0.5 ml of 90% Beckman Tissue Solubilizer for 18 h before radioassay. The ^3H and ^{14}C (or ^{35}S) d.p.m. were calculated by correcting for spillover and quench with efficiencies of 31–33% and 72–73%, respectively.

Isoelectric focusing. Prior to electrofocusing, the samples were chromatographed on a 0.9 cm $\times$ 58 cm column of Sephadex G-25 in 0.02 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. The fractions containing protein were dialyzed exhaustively against water (using 1 mM-phenylmethylsulphonyl fluoride in the first dialysis buffer) and lyophilized. Samples (0.1–0.3 mg of protein) were dissolved in 10% (w/v) sucrose, 2% NP-40, 8 M-urea, and 2 mM-dithioerythritol, heated for 90 s at 100°C, made up to 2% ampholyte, layered on polyacrylamide gels (10 cm $\times$ 0.5 cm) and overlaid with 0.1 ml of 2% ampholyte in 2% NP-40/8 M-urea solution containing 5% (w/v) sucrose. The gels contained 2.9% (w/v) acrylamide/0.15% bisacrylamide, 2% ampholyte (pH ranges 4–6, 5–7 and 3.5–10 in 2:2:1 proportions), 13% (w/v) sucrose, 2% (w/v) NP-40, and 8 M urea and were polymerized with 0.1% NNN'N'-tetramethylethylenediamine and 0.06% ammonium persulphate. The anode solution (bottom) was 0.01 M- H_3PO_4 ; the cathode was 0.02 M-NaOH. The current was adjusted to 0.5 mA/gel and the potential was increased gradually until it reached 230 V at which level it was maintained for an additional 22–24 h. Gels were radioassayed as above.

Gel filtration. Samples were chromatographed on a 1.5 cm $\times$ 64 cm column of Sephadex G-200 by the method of Bornens & Kasper (1973).

Results

Effect of insulin on sodium transport

Insulin stimulates trans-epithelial sodium transport for up to 24 h following administration. In tissues pretreated with cycloheximide, the short circuit current, a direct measure of active sodium transport in this tissue, initially increases after insulin addition but rapidly returns to a level indistinguishable from control (Fig. 1). The early and transient stimulation of short circuit current was observed regardless of the length of time tissues were pre-incubated with inhibitors (up to 3 h) or the concentration of insulin used (1–250 mU/ml).

Effect of insulin on uptake and incorporation of labelled amino acids

The accumulation of ^{14}C -labelled amino acids into the trichloroacetic acid-soluble fraction and in the trichloroacetic acid precipitate of mucosal cells scraped from hemibladders incubated with insulin was measured. Similar studies were also performed

Fig. 1. *Effect of insulin on sodium transport across the toad urinary bladder*

The short circuit current was measured in a pair of hemibladders, one of which was used as control (—) and one which was exposed to 1 μg of cycloheximide/ml (---). After 120 min, insulin (1 mU/ml) was added to the Ringer's solution bathing each tissue.

using isolated MR and G cells that were incubated with insulin after separation. In both cases the rate of uptake and the accumulation of label in the acid precipitate was linear for up to 4 h and was unaltered by insulin administration (results not shown). Insulin had no effect on the rate of aminoisobutyrate uptake (results not shown).

Insulin-induced incorporation of amino acids into specific proteins

We studied proteins from two different types of preparations: (1) mucosal cells prepared from control and insulin-treated bladders, which had been exposed to insulin only while the tissues were intact, or (2) mucosal cells separated prior to treatment with insulin.

In the intact bladder. To examine the effects of insulin on protein synthesis, concentrations of insulin that elicit the maximal physiological response were generally used. The maximal short circuit current response was attained with pig insulin concentrations between 20 and 50 mU/ml (150–400 nM) (Cobb & Scott, 1979). The requirement for such concentrations to obtain a maximal response is presumably related to the observation that mammalian insulin is much less potent in amphibians than is the endogenous hormone (Manery *et al.*, 1977).

Incubation for 1 h: insulin increased the labelling of several soluble proteins in the G cells of intact bladders after 1 h. The electrophoretic profile of a soluble fraction from G cells (Fig. 2a) indicates that the largest increase in the $^{14}\text{C}/^3\text{H}$ ratio corresponds

Fig. 2. Incorporation of radiolabelled amino acids into proteins by G cells after 1 h of exposure of the intact bladder to insulin

Bladders were incubated with insulin (250 mU/ml) and ^{14}C -labelled amino acids, while paired hemibladders were incubated with ^3H -labelled amino acids. After 1 h, mucosal cells were removed, separated into MR and G cell types and fractionated into a soluble and membrane fractions. Proteins were analyzed on SDS/polyacrylamide gels as described in the Materials and methods section. (a) Isotope ratio ($^{14}\text{C}/^3\text{H}$) of the incorporated precursors in each slice of a gel from the soluble fraction; (b) isotope ratio from the gel of the plasma membrane fraction; (c) isotope ratio of the soluble fraction from an identical experiment except that neither incubation included insulin. The molecular weight calibration for all of the SDS/polyacrylamide gels is shown in Fig. 4.

to a protein with an R_F 0.76, or an M_r 16000. The absorbance scan of a stained gel indicated that this protein is quantitatively a minor component of the soluble fraction. The same result was obtained when the radioactive isotopes in the insulin and control baths were reversed. There were no measurable peaks in the isotope ratio in any other subcellular fractions, including the plasma membranes (Fig. 2b), from the tissues exposed to insulin for 1 h. In control versus control experiments the ratio throughout the gel of the soluble fraction was constant (Fig. 2c).

Incubation for 3 h: in three experiments in which the incubation with insulin and labelled amino acids continued for 3 h, none of the proteins of the soluble fraction from the granular cells exhibited a significant deviation in the isotope ratio (Fig. 3b).

Fig. 3. Incorporation of radiolabelled amino acids into proteins by G cells after 3 h of exposure of the intact bladder to insulin

Bladders were incubated with insulin (250 mU/ml) and ^3H -labelled amino acids, while paired hemibladders were incubated with ^{14}C -labelled amino acids. After 3 h, samples were prepared as in Fig. 2 and electrophoresed on SDS/polyacrylamide gels. (a) Isotope ratio in each gel slice of the plasma membrane fraction; (b) ratio from the soluble fraction; (c) ratio of the plasma membrane fraction from an otherwise identical experiment in which the incubation did not include insulin.

However, analysis of the plasma membrane fraction revealed the isotope ratio of one band with an M_r 25000 was increased 2-fold over the baseline value (Fig. 3a). There was also a (ratio) peak (1.8 times the baseline ratio) in the microsomal fraction with an R_F identical to that of the plasma membrane protein. Proteins from the MR cells derived from the same tissues had no peaks in the isotope ratio after either 1 or 3 h of insulin treatment (results not shown).

Induced proteins in separated cells. To reduce the time between insulin treatment and the analysis of fractionated, labelled proteins, we also used suspensions of isolated MR and G cells for the labelling experiments. Cells were treated with a proteinase inhibitor and disruption begun within 15 min after the end of the hormone treatment.

Incubation for 1 h: the electrophoretic profile of the soluble proteins from G cells exposed to insulin for 1 h is shown in Fig. 4. There is one ratio peak in this fraction with a mobility corresponding to M_r 16000. Urea (9M) had no effect on the electrophoretic profile. Using the ratios in the individual gel

Fig. 4. Incorporation of radiolabelled methionine into soluble proteins of isolated G cells after a 1 h exposure to insulin

A suspension of isolated mucosal cells was divided into two aliquots. [³⁵S]Methionine was added to one group, while [³H]methionine and insulin (100 mU/ml) were added to the other group. After 1 h the cells were fractionated and analyzed as in the Materials and methods section. The d.p.m. of each isotope in each gel slice and the resulting ratio from the G cell soluble fraction, as well as the absorbance scan of the stained gel, are plotted. Inset, the molecular weight calibration: A, phosphorylase A; B, glucose oxidase; C, bovine serum albumin; D, ovalbumin; E, carbonic anhydrase; F, chymotrypsinogen; G, myoglobin; H, cytochrome c.

slices as separate data points, a mean and s.d. were computed for each gel. In the gel shown in Fig. 4, these values were 0.20 ± 0.067 ; the ratio (0.67) of the peak (M_r 16 000) is 7 s.d. above the mean. There were no consistent ratio peaks in the plasma membrane fractions of the G cells.

Gel filtration of the soluble fraction on Sephadex G-200, to examine high molecular weight proteins that were poorly resolved in 8% acrylamide gels, confirmed the presence of a ratio peak corresponding to a molecular weight of 16 000–17 000, as well as two other ratio peaks corresponding to proteins of higher molecular weight (Fig. 5). The SDS/polyacrylamide gel of this sample also had more than one ratio peak. Ratio peaks in addition to

Fig. 5. Gel filtration of the soluble proteins from G cells exposed to insulin for 1 h

The soluble proteins from an experiment similar to that described in Fig. 4 were chromatographed on Sephadex G-200 as described in the Materials and methods section. Shown are the d.p.m. and the isotope ratio of each fraction. Inset, the molecular weight calibration of the column: A, ferritin; B, bovine serum albumin; C, γ -globulin, heavy chain; D, γ -globulin, light chain; E, cytochrome c.

the one at M_r 16 000 were found in four out of ten experiments.

Isoelectric focusing (Fig. 6) of the G cell soluble protein sample reveals that the induced proteins had isoelectric points between 4.0 and 4.5. Since only one peak was resolved by electrophoresis in an SDS/polyacrylamide gel (Fig. 4), the focusing gel may have resolved several different induced proteins with nearly identical electrophoretic mobilities; alternatively, the multiple peaks may be artifacts of the focusing conditions.

Incubation for 3 h: the M_r 16 000 induced protein was also observed in the soluble fraction of G cells exposed to insulin for 3 h. The G cell plasma membrane fraction also revealed a ratio peak at R_F 0.64 (corresponding to an induced protein of M_r 25 000), 3.9 s.d. greater than the mean ratio (Fig. 7). This ratio peak, observed in all of five experiments in separated cells, was also found in experiments with intact bladders (Fig. 3).

Incubation for 2 h: we have also studied the labelling of cells exposed to insulin for 2 h, a period of transition between the transient and the sustained sodium transport response. There are two ratio peaks, one the M_r 16 000 soluble protein, in two of four experiments; in the plasma membranes, a ratio

Fig. 6. Isoelectric focusing of the soluble proteins from G cells exposed to insulin for 1 h

The soluble proteins from the experiment described in Fig. 4 were analysed by isoelectric focusing as described in the Materials and methods section. The d.p.m. and the isotope ratio are plotted as a function of slice number, along with the pH gradient.

Fig. 7. Incorporation of radiolabelled methionine into plasma membrane proteins of isolated G cells after a 3 h exposure to insulin

A suspension of isolated mucosal cells was divided into two aliquots. [^3H]Methionine was added to one group, while [^{35}S]methionine and insulin (10 mU/ml) were added to the other group. After 3 h the fractions were prepared as in Fig. 4. Shown are the d.p.m. of each isotope, the isotope ratio and the absorbance scan of the stained gel from the G cell plasma membrane fraction.

peak corresponding to M_r 25 000 was apparent in half of the experiments.

Effect of insulin concentration

To relate the appearance of the induced proteins to insulin concentration, we compared the effects of 1, 10 and 100 mU/ml (7 nM, 70 nM and 700 nM) insulin on the induction of the M_r 16 000 soluble protein in G cells after 1 h of insulin exposure. The largest ratio peak (4.4 s.d. above the mean ratio) was found at a saturating concentration, 100 mU/ml (Fig. 8). At 10 mU/ml, a near maximal insulin concentration, the ratio peak at M_r 16 000 is 3.2 s.d. above the mean ratio. An increased

Fig. 8. Effect of insulin concentration on the appearance of the M_r 16 000 ratio peak from the G cell soluble fraction

The experiments were performed as described in Fig. 4. Insulin concentration: (a) 100 mU/ml, (b) 10 mU/ml, (c) 1 mU/ml, (d) no insulin.

labelling of the M_r 16000 protein is also observed at 1 mU/ml, a hormone concentration that causes approximately 40% of the maximal increase in short circuit current. The induced peak is 3.8 s.d. above the mean. A control experiment (no insulin) is included in Fig. 8 for comparison.

Effects of cycloheximide

The hormone-induced increases in the ratio of radioactively labelled proteins could be the result of either an increase in the rate of protein synthesis or a decrease in degradation, or both, in treated tissues. Therefore, we compared the effects of 1 h of insulin treatment on the labelling of the soluble fraction of G cells pre-incubated for 1 h with cycloheximide. Suppression by cycloheximide of protein synthesis would be expected to produce an isotope ratio profile similar to that obtained in an experiment pairing insulin and control, if the main effect of the hormone is on protein synthesis. Fig. 9 shows that we

Fig. 9. Effect of cycloheximide on the appearance of the M_r 16000 insulin-induced ratio peak from the G cell soluble fraction

Cycloheximide was added to one of two suspensions of G cells. After 1 h, insulin (100 mU/ml) and [^{35}S]methionine were added to this group of cells, while insulin and [^3H]methionine were added to the other group of cells. The rest of the experiment was performed as described in Fig. 4. The d.p.m. of each isotope and the isotope ratio in the low molecular weight region of the gel are shown.

obtained this result, with a ratio peak 3.2 s.d. above the mean. Another factor in support of an apparent increase in the rate of synthesis of the proteins, at least after 1 h of treatment, is the relatively brief labelling time. With a short incubation time the assumption might be made that changes in labelling are due solely to increases in synthesis (Ivarie & O'Farrell, 1978).

Time course of appearance of insulin-induced ratio peaks in separated cells

Since the determination of protein induction was based on changes in the isotope ratio, it was not possible to use the absolute magnitude of the changes in the isotope ratio to follow the time course of induction in experiments not performed on the same day. Thus, we used the frequency of appearance of ratio peaks (defining peaks as 2 s.d. above the baseline) at various times after exposure to insulin as an index of their time course of induction. Fig. 10 compares the time courses of induction of the M_r 16000 soluble protein and the M_r 25000 membrane protein with the time course of that fraction of hormone-stimulated sodium transport sensitive to pretreatment with cycloheximide, actinomycin D and puromycin. The time course of the appearance of these proteins in the mucosal cells is consistent with, but does not prove, a causal relationship between their appearance and inhibitor-sensitive insulin-stimulated sodium transport.

Fig. 10. Time course of induction of the M_r 16000 soluble protein, the M_r 25000 membrane protein and comparison with the time course of the inhibitor-sensitive component of the insulin-stimulated short circuit current

The time courses of induction of the soluble and the plasma membrane protein, determined as described in the Materials and methods section, are plotted in the centre and right-hand panels, respectively. The inhibitor-sensitive component of insulin-stimulated short circuit current is plotted on the left. These values were obtained by combining the average short circuit current ($n=3-6$) which could be inhibited by all three inhibitors, actinomycin D, cycloheximide and puromycin. Data for actinomycin D and puromycin were taken from Cobb *et al.* (1981).

Discussion

A number of epithelia (Herrera, 1963, 1965; Crabbé & Francois, 1967; Nizet *et al.*, 1971), including the distal nephron of the human kidney (DeFronzo *et al.*, 1975, 1976), respond to insulin by increasing active sodium transport. These and previous studies (Cobb & Scott, 1979) confirm that the long-term effect of insulin on sodium transport, i.e., that persisting after the first 1 h, is sensitive to inhibitors of protein and RNA synthesis. Prior to examining the effects of insulin upon the labelling of tissue proteins, we demonstrated that the hormone has no effects upon the uptake of labelled amino acids by the mucosal cells, either in the intact tissue or in suspension. Although insulin has no effect on total protein synthesis in either cell type, it apparently induces the synthesis of several specific proteins in G mucosal cells in amounts too small to be detected in the total protein pellet. Analysis of the isotope ratio in dual-label experiments indicates that consistently an M_r 16000 protein, and occasionally two to three other proteins, in the soluble fraction of G cells is differentially labelled after one hour of exposure to physiological levels of insulin. After 2 h of insulin treatment, and more clearly after 3 h, an elevated isotope ratio indicative of a hormone-induced protein (M_r 25000) is also observed in the plasma membrane fraction.

The M_r 16000 protein may be identical to the insulin-induced protein reported by Benjamin & Singer (1974). The different M_r (12000) estimated by those investigators using a different electrophoretic method could be within the range of experimental error. The molecular weights of at least two of the insulin-induced proteins in G cells are similar to the molecular weights of some proteins that we have previously found to be induced by aldosterone in the MR cells of this tissue (Scott & Sapirstein, 1975; Scott *et al.*, 1978). In particular we have frequently found a soluble protein of near M_r 16000 induced by both hormones. The M_r 180000 insulin-induced, soluble protein which appears in certain experiments is similar in size to one of the aldosterone-induced plasma membrane proteins. The relationships and identities of these proteins are unknown, but the appearance of possibly similar proteins induced by both hormones may suggest a similarity in the mechanisms by which the hormones increase sodium transport.

Although a direct relationship between the synthesis of insulin-induced proteins and the stimulation of sodium transport remains to be established, these studies suggest a correlation between specific protein synthesis and increased transport. The synthesis of the induced proteins, as well as the long-term stimulation of sodium transport, can be blocked by pretreatment with cycloheximide.

Furthermore, at least one of the proteins appears to be translated from RNA from insulin-treated cells (Cobb *et al.*, 1981). Although other explanations have not been ruled out, this suggests that the synthesis of this protein may be regulated by insulin at the level of transcription. In addition, the time course of increased appearance of the proteins is appropriate for their possible participation in augmenting sodium transport in the second, long-term phase of the insulin effect on transport. Finally, the dependence of protein induction on insulin concentration is comparable to that of the transport response. These findings, along with the absence of any other detectable effects of insulin on the epithelial cells (Cobb *et al.*, 1981), all are consistent with the hypothesis that the induced proteins are related to enhanced sodium transport.

The mechanism we have proposed previously for aldosterone action in the toad bladder epithelium involves the binding of aldosterone by MR cells, the stimulation of protein synthesis in the MR cell, and thereby, enhanced sodium transport by the tissue (Scott & Sapirstein, 1975; Scott *et al.*, 1978). We now present evidence that insulin, which apparently regulates sodium transport by a mechanism independent of aldosterone, acts primarily or solely upon the G cells, perhaps by inducing the synthesis of proteins in the cells. Siegel & Civan (1976) suggested, on the basis of their studies of the changes in driving force of sodium transport caused by these two hormones, that there may be two parallel populations of cells transporting sodium that are differentially stimulated by aldosterone and insulin. Our results suggest that the MR cells, which are apparently the aldosterone target cells, and the G cells, which evidently are the insulin target cells, may be the two separately regulated, transporting cell populations.

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