

Insulin Regulation of Na/K Pump Activity in Rat Hepatoma Cells

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SUMMARY

Insulin rapidly increases Na/K pump activity in HTC rat hepatoma cells in tissue culture, as measured by the ouabain-sensitive influx of the potassium analogue $^{86}\text{Rb}^+$. Increased influx is observed within minutes and is maximal (70% above control) within 1–2 h. The effect appears to be mediated by the insulin receptors, as: (1) the concentration dependence on insulin is identical to that for insulin induction of tyrosine aminotransferase and stimulation of 2-aminoisobutyric acid transport, (2) proinsulin is 6% as potent as insulin, and (3) the effect is blocked by anti-receptor antibodies. The early stimulation of potassium influx is not blocked by cycloheximide and is not associated with an increased number of pump sites as measured by ^3H -ouabain binding. The insulin effect is blocked by amiloride, which blocks sodium influx, and is mimicked by the sodium ionophore monensin, which increases sodium influx and intracellular accumulation. Insulin also rapidly increases the initial rate of $^{22}\text{Na}^+$ influx, suggesting that insulin may enhance Na/K pump activity, in part, by increasing intracellular sodium concentration. Incubation of HTC cells with insulin for 24 h causes complete unresponsiveness to the insulin induction of transaminase and stimulation of amino acid transport, a phenomenon mediated by postbinding mechanisms. In contrast, similar incubation with insulin does not cause unresponsiveness to the insulin stimulation of Na/K pump activity. Therefore, the site of regulation of responsiveness to insulin must be distal to, or separate from, those events causing stimulation of ion fluxes. *DIABETES* 33:428–434, May 1984.

Insulin causes a two- to threefold induction of tyrosine aminotransferase in HTC rat hepatoma cells in tissue culture^{1,2} and, in dexamethasone-treated cells, a five- to tenfold increase in rate of transport of the nonmetabolizable amino acid 2-aminoisobutyric acid.^{3–5} In the continued presence of insulin, however, these responses are lost and

the cells become completely insensitive to even high concentrations of insulin.⁴ Although insulin downregulates insulin receptors, we have shown that insulin-induced unresponsiveness is mediated by as yet undefined postbinding mechanisms.⁵ To study these postbinding regulatory events, we have characterized the insulin stimulation of Na/K pump activity in HTC cells, hoping to use this early membrane effect of insulin as a marker of rapid changes in insulin responsiveness.

The Na/K pump mediates the active influx of K^+ into cells and the extrusion of Na^+ , and its activity is known to be modulated by hormones.⁶ Specifically, insulin stimulation of Na/K pump activity has been reported in a variety of mammalian and amphibian cells and tissues.⁷ We report here that, in HTC cells, insulin rapidly increases the ouabain-sensitive influx of the potassium analogue $^{86}\text{Rb}^+$, a marker of Na/K pump activity. The stimulation is seen within minutes and is maximal at 1 h. The effect is mediated by the insulin receptor, and appears to be at least partially secondary to an increase in Na^+ influx. In contrast to the insulin stimulation of transaminase induction and amino acid transport described above, the early stimulation of Na/K pump activity by insulin does not appear to be lost in cells previously incubated with insulin.

MATERIALS AND METHODS

Cell culture. HTC cells were grown in monolayer culture in antibiotic-free Eagle's Minimal Essential Medium, alpha modification, supplemented with 5% calf serum and 5% fetal bovine serum. Cells were collected by trypsinization and replated in 35-mm tissue culture dishes for uptake measurements as described below. Before hormone treatment and uptake measurements, confluent cultures were incubated for 18 h in serum-free medium modified to contain

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50 mM tricine, 0.5 g/L sodium bicarbonate, and 0.1% bovine serum albumin (induction medium). This medium contains 5 mM potassium.

$^{86}\text{Rb}^+$ uptake. Uptake measurements were carried out at 37°C for 20 min in 1.1 ml of induction medium containing 1.5 $\mu\text{Ci}/\text{dish}$ of $^{86}\text{RbCl}$. Parallel dishes were incubated with 1–2 mM ouabain to determine the ouabain-sensitive component of rubidium uptake. Inhibition of Na/K pump activity was the same whether ouabain was added 5 min before, 1 min before, or at the same time as $^{86}\text{Rb}^+$. The assay was terminated by placing the dishes on ice and aspirating the medium. The cell layer was washed three times with ice-cold 0.1 M MgCl_2 . Preliminary experiments indicated that the amount of cell-associated $^{86}\text{Rb}^+$ was constant after 2–5 washes with MgCl_2 . After the MgCl_2 washes, the dishes were incubated for 30 min at 4°C with 1 ml of 5% trichloroacetic acid. Acid-soluble radioactivity was then determined by Cerenkov radiation in a Packard Tri-Carb scintillation spectrometer with an efficiency of approximately 27%. The amount of cell protein per dish was measured by the method of Lowry et al.⁹ after dissolving the cells in 1 N NaOH.

Potassium influx was calculated as:

$$\text{K}^+ \text{ influx (nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}) = \frac{(\text{total intracellular } ^{86}\text{Rb}^+ \text{ cpm}) (\text{extracellular K}^+ / \text{dish})}{(\text{total } ^{86}\text{Rb}^+ \text{ cpm/dish}) (20 \text{ min}) (\text{mg protein/dish})}$$

$^{22}\text{Na}^+$ uptake. $^{22}\text{Na}^+$ uptake was measured as described for $^{86}\text{Rb}^+$ uptake, except that 1.5 mM ouabain was included in each incubation to prevent loss of accumulated intracellular sodium by the Na/K pump. $^{22}\text{NaCl}$ (0.3 μCi) was added to each dish, and TCA-soluble radioactivity was assayed in a Beckman gamma spectrometer with an efficiency of ap-

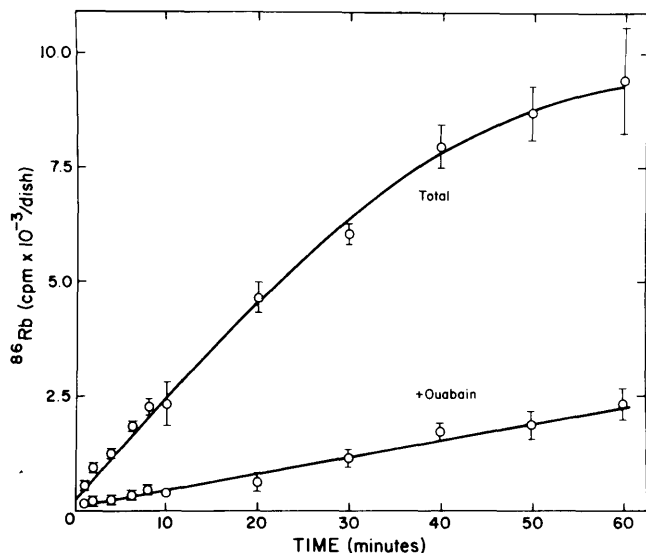


FIGURE 1. Time course of $^{86}\text{Rb}^+$ uptake. Monolayer cultures of HTC cells in 35-mm dishes were incubated for 18 h in serum-free medium, after which 1.5 μCi $^{86}\text{Rb}^+$ plus 4 $\mu\text{g}/\text{ml}$ insulin was added to each dish in the presence or absence of 1 mM ouabain. At the times indicated, quadruplicate dishes were placed on ice, the medium aspirated, and the cell layer washed three times with ice-cold, 0.1 M MgCl_2 . TCA-soluble radioactivity was assayed in a scintillation spectrometer. Each datum represents the mean \pm SD of four dishes.

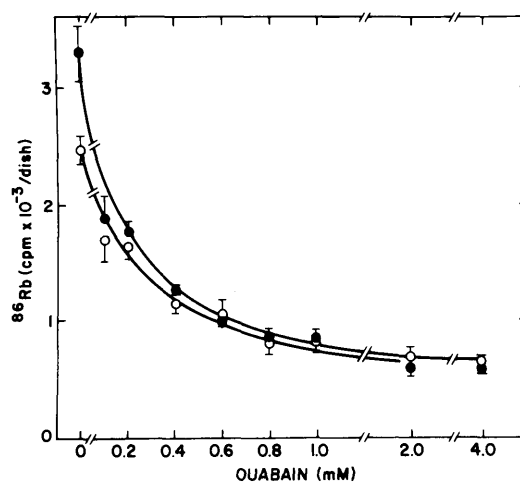


FIGURE 2. Ouabain inhibition of $^{86}\text{Rb}^+$ uptake. Monolayer cultures of HTC cells were incubated for 1 h in the presence (closed symbols) or absence (open symbols) of 4 $\mu\text{g}/\text{ml}$ insulin. $^{86}\text{Rb}^+$ (0.5 $\mu\text{Ci}/\text{dish}$) plus ouabain at the concentrations indicated was then added and the incubation continued for another 20 min. $^{86}\text{Rb}^+$ uptake was assayed as described in MATERIALS AND METHODS. Each datum represents the mean \pm SD of quadruplicate dishes.

proximately 55%. Pilot experiments indicated that $^{22}\text{Na}^+$ uptake was linear for 8–10 min and had reached a steady state by 20 min.

^3H -ouabain binding. Because of the low affinity of ouabain binding to rodent cells, it was not possible to measure binding under the same conditions as rubidium and sodium uptake. Therefore, ouabain binding experiments were carried out in potassium-free conditions.^{10,11} Confluent cultures of HTC cells in 60-mm dishes were incubated in serum-free induction medium for 18 h. After further incubation with or without 4 $\mu\text{g}/\text{ml}$ insulin, the dishes were washed twice with potassium-free, phosphate-buffered saline, pH 7.4, and then incubated at 37°C for 30 min with 2 ml of fresh potassium-free phosphate-buffered saline, with or without insulin, and with or without 10 mM ouabain (to measure nonspecific binding). ^3H -ouabain (2 μM , 6 $\mu\text{Ci}/\text{dish}$) was added to each dish and the incubation continued for another 60 min at 37°C. The medium was aspirated and the cells washed 4 times with ice-cold, potassium-free, phosphate-buffered saline, and lysed in 1 N NaOH. Radioactivity was assayed in Liquefluor-toluene in a liquid scintillation spectrometer. Pilot experiments indicated that ouabain binding reached steady state within 30–45 min under these conditions.

Materials. Tissue culture media and sera were obtained from Grand Island Biological Company, Grand Island, New York. Monocomponent porcine insulin (lot #615-07J-256) and proinsulin were gifts from Dr. Ronald Chance, Eli Lilly and Company, Indianapolis, Indiana. Anti-insulin receptor antiserum was kindly provided by Drs. C. R. Kahn and G. King of the Joslin Diabetes Center, Boston, Massachusetts. An IgG fraction was prepared by protein A-Sepharose chromatography. Monensin was kindly provided by Dr. M. Schlafer of this institution and Dr. R. L. Hamill of Eli Lilly and Company. Amiloride was donated by Dr. D. Dawson of the University of Michigan. $^{86}\text{RbCl}$ (1–10 Ci/g), carrier-free $^{22}\text{NaCl}$ (>100 Ci/g), and ^3H -ouabain (18 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts.

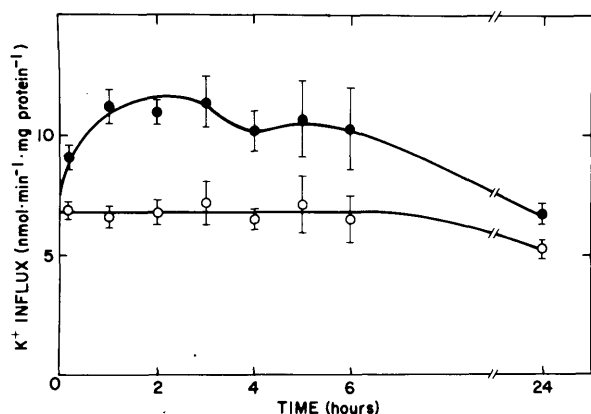


FIGURE 3. Time course of insulin induction of potassium influx. Monolayer cultures of HTC cells in 35-mm dishes were incubated in the presence (closed symbols) or absence (open symbols) of 4 $\mu\text{g/ml}$ insulin for the times indicated in the figure. At each time point, ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured. Each datum represents the mean \pm SEM from 3 to 9 experiments at each time point.

Ouabain and cycloheximide were purchased from Sigma, St. Louis, Missouri.

RESULTS

Insulin stimulation of Na/K pump activity. The time course of $^{86}\text{Rb}^+$ uptake in insulin-treated HTC cells is shown in Figure 1. Uptake is linear for 30–40 min; therefore, all subsequent measurements were carried out for 20 min to insure accurate measurement of initial rates of rubidium uptake. The time course of $^{86}\text{Rb}^+$ uptake in control cells is similar. In the presence of 1 mM ouabain, rubidium uptake is inhibited by 75–80%. This ouabain-sensitive component of rubidium uptake reflects Na/K pump activity.⁶

Figure 2 illustrates the concentration dependence of the ouabain inhibition of $^{86}\text{Rb}^+$ uptake in control cells and cells

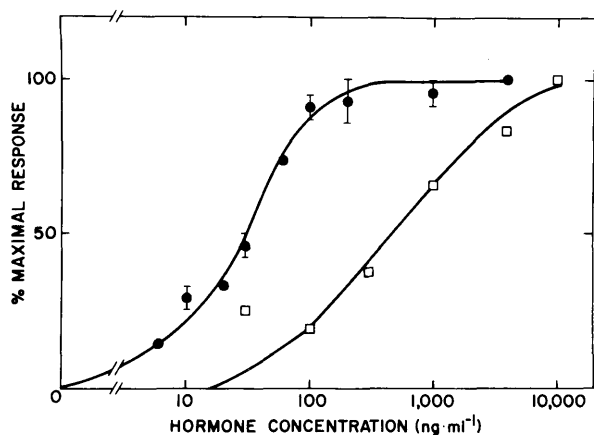


FIGURE 4. Concentration dependence of the insulin stimulation of $^{86}\text{Rb}^+$ transport. Monolayer cultures were incubated for 1 h in the presence of varying concentrations of insulin or proinsulin. $^{86}\text{Rb}^+ \pm 2$ mM ouabain was added to each dish, and ouabain-sensitive $^{86}\text{Rb}^+$ uptake measured over a further 20-min incubation. The data are presented as the percent of maximal response obtained with insulin. Closed circles represent insulin and open squares proinsulin. Insulin data points with error bars represent the mean \pm SEM from five experiments; data points without error bars represent the mean of two experiments. The proinsulin data represent the mean of two experiments. Maximal stimulation by insulin was $70 \pm 10\%$ (mean \pm SEM) and the mean maximal stimulation by proinsulin was 90%.

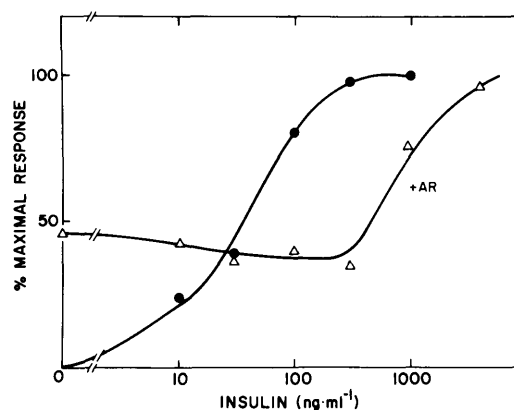


FIGURE 5. The effect of anti-insulin receptor antibodies on the concentration dependence of insulin stimulation of $^{86}\text{Rb}^+$ uptake. Monolayer cultures of HTC cells were incubated for 1 h in the presence of various concentrations of insulin in the presence (open triangles) or absence (closed circles) of 85 $\mu\text{g/ml}$ anti-insulin receptor IgG, a concentration sufficient to inhibit ^{125}I -insulin binding by 75%. Uptake of $^{86}\text{Rb}^+$ over a 20-min period was assayed and ouabain-sensitive potassium influx calculated. The data are expressed as percent of maximal response and represent the mean values of triplicate determinations. Maximal stimulation of $^{86}\text{Rb}^+$ uptake by insulin in this experiment was 70%.

incubated with insulin. Rubidium uptake is half-maximally inhibited at approximately 0.2–0.3 mM ouabain; maximal inhibition is obtained at concentrations >1 mM.

The time course of insulin stimulation of ouabain-sensitive potassium influx is shown in Figure 3. Insulin (4 $\mu\text{g/ml}$) causes a rapid increase in Na/K pump activity, apparent within the first 20 min of incubation with insulin. The insulin stimulation is maximal after 1 h of incubation with the hormone. In most experiments, the initial rate of potassium influx appears to fall slightly after 3–4 h of incubation with insulin, and then shows a secondary rise. After 24 h incubation with insulin, the initial rate of potassium influx has fallen to near that seen in cells that have not been exposed to insulin.

The magnitude of the insulin-stimulated increase in pump activity, based on 30 experiments, was $69 \pm 3\%$. Insulin has no consistent effect on ouabain-insensitive rubidium influx (see Figure 2), suggesting that its entire effect is on Na/K pump activity. Insulin also has no effect on rubidium efflux (data not shown). Incubation of cells with the glucocorticoid dexamethasone has no effect on rubidium uptake, nor does it alter the insulin stimulation of Na/K pump activity (data not shown).

Role of the insulin receptor in the insulin stimulation of Na/K pump activity. The concentration dependence of the insulin and proinsulin stimulation of ouabain-sensitive rubidium transport is shown in Figure 4. Half-maximal stimulation of Na/K pump activity is observed at 30 ng/ml (5 nM) insulin, the same concentration that causes half-maximal induction of tyrosine aminotransferase and half-maximal stimulation of AIB transport in HTC cells.^{1,5} Proinsulin is approximately 6% as effective as insulin.

Figure 5 shows the effect of anti-insulin receptor IgG, at a concentration sufficient to inhibit ^{125}I -insulin binding by 75%, on the concentration dependence of insulin stimulation of ouabain-sensitive rubidium uptake. Anti-receptor antibodies, as expected, have insulinomimetic properties¹² and stimulate rubidium transport. However, they also shift the insulin dose-response curve to the right, as would be expected if

the insulin stimulation of rubidium uptake is mediated by the insulin receptor.

Mechanism of insulin stimulation of Na/K pump activity.

Insulin could increase Na/K pump activity either by increasing the number of active pump units on the cell membrane or by increasing their activity (e.g., by increasing their affinity for sodium⁷ or by increasing intracellular sodium concentration^{13,14}) or by some combination of these mechanisms.

We first investigated the requirement for concomitant protein synthesis by examining the insulin stimulation of ouabain-sensitive rubidium transport in the presence or absence of cycloheximide. At a concentration of 0.1 mM, cycloheximide inhibits amino acid incorporation into protein in HTC cells by more than 98% and completely abolishes the insulin stimulation of AIB transport and transaminase induction.³ As shown in Figure 6, inhibition of protein synthesis blunts, but does not completely block, insulin stimulation of rubidium transport. At 1 h, insulin stimulated ⁸⁶Rb⁺ uptake by 75% in the absence of cycloheximide and by 45% in its presence, a reduction of approximately 40%. After 2 h incubation with cycloheximide, the rate of rubidium uptake in both insulin-treated and control cells declined in parallel. These data, together with the rapidity of the insulin stimulation of Na/K pump activity, argue against the synthesis of new pump units as the mechanism of insulin stimulation.

Insulin might still increase the number of active pumps by unmasking cryptic pump units.¹⁵ We attempted to assess the number of Na/K pump units on the membrane by ³H-ouabain binding studies. The low affinity of ouabain binding to rodent cells precluded measurement of binding under the same conditions as assay of rubidium transport, i.e., in medium containing 5 mM potassium. Therefore binding studies were carried out in potassium-free medium as described in MATERIALS AND METHODS. The data shown in Table 1 indicate that the amount of tracer ³H-ouabain bound by HTC cells is not increased after either 1 h or 5 h incubation with insulin. Scatchard analysis of ouabain binding data suggests the pres-

TABLE 1
Effect of insulin on ouabain binding

Time of incubation	³ H-ouabain binding (fmol · mg protein ⁻¹)	
	Control	Insulin
1 h	870 ± 84	948 ± 114
5 h	1056 ± 135	869 ± 69

Binding of ³H-ouabain to HTC cells was measured as described in MATERIALS AND METHODS after incubation with insulin for 1 or 5 h. Data represent the mean ± SEM from four experiments.

ence, in both control and insulin-treated cells, of a binding site with an apparent K_D of approximately 5 μM and a B_{max} of 3.5 pmol · mg⁻¹ protein, or approximately 500,000 sites/cell (data not shown). Thus, there is no evidence that insulin increases the number of pump units on HTC cells.

Stimulation of the activity of the Na/K pump by increasing intracellular Na⁺ concentration has been reported in a number of cell types in tissue culture.¹³ Increased influx of Na⁺, caused by the carboxylic polyether ionophore monensin,¹⁶ also enhances the activity of the Na/K pump in HTC cells. As shown in Figure 7, monensin causes a concentration-dependent increase in the initial rate of potassium influx. The effect is half-maximal at approximately 0.7 μg/ml (1 μM) and maximal at 5 μg/ml. Monensin causes a parallel increase in the initial rate, as well as steady-state accumulation, of ²²Na⁺ in ouabain-treated cells (data not shown).

As in other cell types,^{17,18} a significant component of sodium uptake in HTC cells can be blocked by the diuretic amiloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide). Amiloride causes a maximal inhibition (64%) of ²²Na⁺ uptake in HTC cells at a concentration of 100 μg/ml (0.44 mM); half-maximal inhibition is achieved at approximately 0.1 μg/ml (data not shown). Figure 8 shows the effect of 0.5 mM amiloride on basal and insulin-stimulated potassium influx in HTC cells. Although amiloride causes little

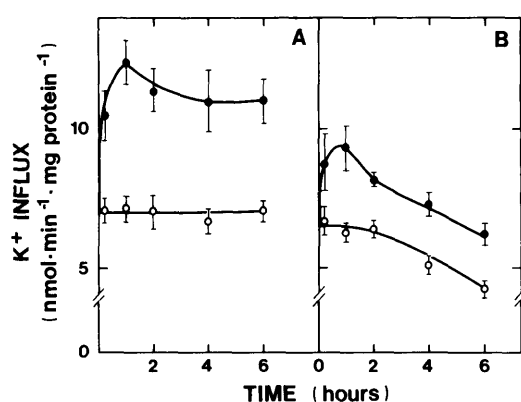


FIGURE 6. Effect of cycloheximide on the time course of insulin stimulation of potassium influx. Monolayer cultures were incubated in the presence or absence of 4 μg/ml insulin for the times indicated. At each time point, ouabain-sensitive ⁸⁶Rb⁺ uptake was measured. A parallel set of cultures was incubated with 0.1 mM cycloheximide for 30–60 min before the addition of insulin. Data are expressed as ouabain-sensitive potassium influx; each datum represents the mean ± SEM from three experiments. Panel A, without cycloheximide; panel B, with cycloheximide. Open circles, control; closed circles, insulin.

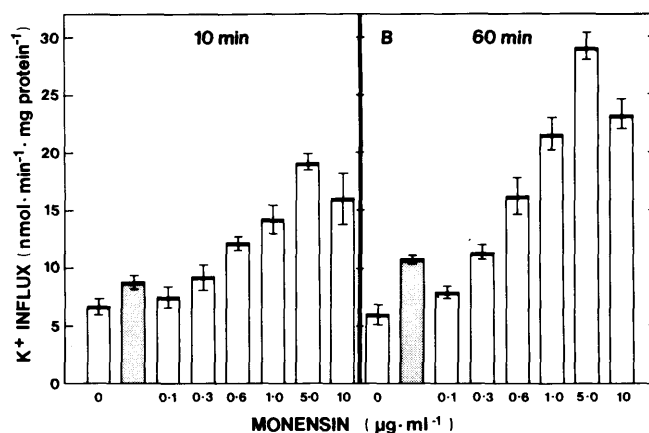


FIGURE 7. Effect of monensin on ouabain-sensitive potassium influx. Monolayer cultures of HTC cells were incubated for a total of 70 min with 4 μg/ml insulin (shaded bars) or monensin (open bars) at the concentrations indicated. All dishes received ethanol to a final concentration of 0.2%. Ouabain-sensitive ⁸⁶Rb⁺ uptake was measured at 0 and 50 min; the times shown on the figure represent the duration of exposure to insulin or monensin at the midpoint of the ⁸⁶Rb⁺ uptake. Data represent mean ± SD of triplicate assays.

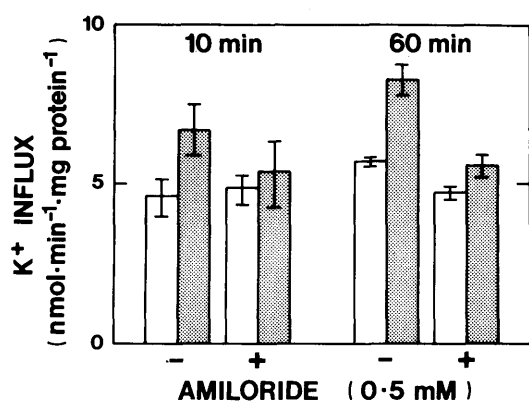


FIGURE 8. Effect of amiloride on the insulin stimulation of potassium influx. Monolayer cultures of HTC cells were incubated for 15 min with or without 0.5 mM amiloride and for an additional 70 min with (shaded bars) or without (open bars) 4 $\mu\text{g/ml}$ insulin. Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured at the time of insulin addition and after 50-min incubation with insulin. The times shown on the figure represent the duration of insulin exposure at the midpoint of the $^{86}\text{Rb}^+$ uptake. Data represent the mean \pm SD of quadruplicate assays.

inhibition of basal potassium influx, it markedly inhibits (by approximately 70%) the insulin-stimulated increase in potassium influx. Thus, the activity of the Na/K pump in HTC cells can be enhanced by increasing sodium influx, and the insulin-induced stimulation of pump activity can be blocked by inhibiting sodium influx.

Direct evidence that insulin stimulates the initial rate of uptake of $^{22}\text{Na}^+$ is shown in Figure 9, which represents the pooled results from three experiments. In the presence of 1.5 mM ouabain, insulin causes a rapid stimulation of sodium influx, apparent within 2–5 min and maximal after 20 min. Taken together, these data suggest that insulin enhances Na/K pump activity, at least in part, by increasing intracellular Na^+ concentration secondary to an increased rate of amiloride-sensitive Na^+ uptake.

Lack of desensitization of insulin stimulation of potassium influx. Incubation of HTC cells for 24 h with 4 $\mu\text{g/ml}$ insulin causes virtually complete unresponsiveness to the insulin stimulation of amino acid transport and induction of transaminase.⁴ As shown in Figure 10, similar treatment does not completely prevent the insulin stimulation of the early increase in ouabain-sensitive rubidium influx. Cultures were incubated for 24 h in the absence or presence of 4 $\mu\text{g/ml}$ insulin, at which time fresh insulin (at that same concentration) was added to half of each set of cultures, and the time course of insulin stimulation of ouabain-sensitive rubidium influx was assayed over the next 6 h. The magnitude of the early effect of insulin is decreased in cells previously incubated with insulin (Figure 10, panel B) relative to that seen in cells that had not previously been incubated with insulin (Figure 10, panel A), but the response is clearly not lost. In the three experiments shown in Figure 10, the magnitude of insulin stimulation of ouabain-sensitive rubidium uptake in control cells was $80 \pm 11\%$; that in cells previously incubated for 24 h with insulin was $42 \pm 9\%$, or approximately half that of the control cells. The maximal rate of insulin-stimulated potassium influx, however, is the same in cells previously incubated with or without insulin; the diminished magnitude of stimulation in the former is largely a function

of the higher basal rate of uptake in the cells previously incubated with insulin.

We have previously reported that insulin causes downregulation of insulin receptor number.⁵ This downregulation causes the expected rightward shift in the dose-response curve for the insulin stimulation of ouabain-sensitive rubidium uptake in cells previously incubated with insulin. The maximal rate of insulin-stimulated rubidium uptake, however, is the same as in cells previously incubated without insulin (data not shown).

DISCUSSION

We report here that insulin stimulates a 70% increase in the initial rate of ouabain-sensitive potassium influx in HTC cells. Increased influx is seen within minutes and is maximal within 1–2 h incubation with insulin.

The effect appears to be mediated by insulin receptors as indicated by the concentration dependence of the insulin stimulation, which is identical to that of insulin induction of tyrosine aminotransferase and stimulation of 2-aminoisobutyric acid transport, by the relative potencies of proinsulin and insulin, and by the inhibition of the insulin effect by anti-insulin receptor antibodies. HTC cells also have receptors for the insulin-like growth factors IGF-I and IGF-II, as demonstrated by affinity cross-linking and competition binding studies. However, the affinity of insulin for binding to the IGF-I receptor is $<0.5\%$ of that for binding to the insulin receptor, and insulin does not bind to the IGF-II receptor even at 10 $\mu\text{g/ml}$. Furthermore, 85 $\mu\text{g/ml}$ of the anti-insulin receptor IgG used in these experiments (B-8, patient H.H.) inhibited ^{125}I -IGF-I binding to HTC cells by $<10\%$ and did not inhibit ^{125}I -MSA binding.¹⁹ Therefore, we conclude that the insulin stimulation of potassium influx is mediated by insulin receptors rather than by IGF receptors.

The insulin stimulation of pump activity during the first hour is blocked by amiloride, which blocks sodium influx, and is mimicked by the ionophore monensin, which increases sodium influx and intracellular accumulation. Insulin rapidly

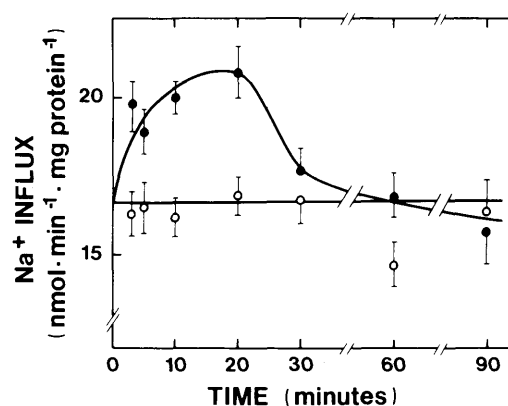


FIGURE 9. Time course of insulin stimulation of $^{22}\text{Na}^+$ influx. Monolayer cultures of HTC cells were incubated with (closed symbols) or without (open symbols) 4 $\mu\text{g/ml}$ insulin for the times indicated. Ouabain was added to quadruplicate dishes to a final concentration of 1.5 mM, and 5 min later 0.3 μCi $^{22}\text{Na}^+$ was added and the incubation continued for an additional 6 min to measure initial rates of $^{22}\text{Na}^+$ uptake. Data are plotted at the midpoint of the $^{22}\text{Na}^+$ uptake, and represent the mean \pm SEM of 12 assays from three experiments, except for the 90-min (two experiments) and 5-min (one experiment) time points.

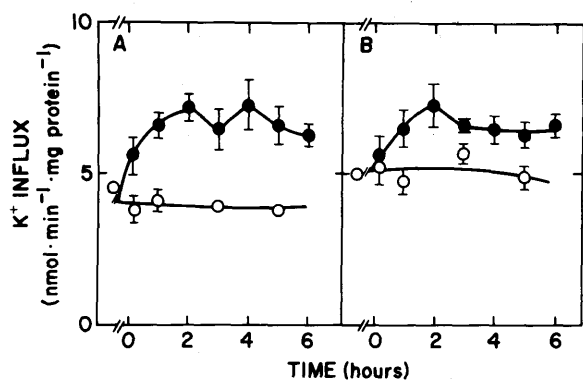


FIGURE 10. Lack of desensitization of insulin stimulation of potassium influx. Monolayer cultures were incubated for 24 h in the absence (panel A) or presence (panel B) of 4 $\mu\text{g/ml}$ insulin. Fresh insulin (4 $\mu\text{g/ml}$) was added to half of each set of cultures (closed symbols). At the times indicated, the initial rate of ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured. Each datum with error bars represents the mean \pm SEM of three or four experiments; data without error bars represent the mean of two experiments.

increases the initial rate of $^{22}\text{Na}^+$ influx, suggesting that insulin may enhance Na/K pump activity in part by increasing intracellular sodium concentration secondary to an increased rate of sodium influx. However, the insulin stimulation of $^{22}\text{Na}^+$ is evanescent, whereas potassium influx remains elevated for several hours. Therefore, it is unlikely that increased intracellular Na^+ can account for the sustained insulin effect on Na/K pump activity.

The early stimulation of potassium influx is blunted, but not completely prevented, by inhibition of protein synthesis with cycloheximide. The possible requirement for protein synthesis in the sustained stimulation by insulin could not be assessed because K^+ influx declined in parallel in both control and insulin-treated cultures after 2 h incubation with cycloheximide. However, we found no evidence for an insulin effect on the number of pump sites, as assessed by ^3H -ouabain binding, even after 5 h incubation with insulin.

Insulin stimulates a 30% increase in rubidium uptake in Fao hepatoma cells in tissue culture. However, since these cells were selected for ouabain resistance, it was not possible to determine whether the insulin effect was on ouabain-sensitive, pump-mediated uptake.²⁰ In freshly isolated rat hepatocytes, insulin also stimulates a modest (18%) increase in ouabain-sensitive rubidium uptake. As in HTC cells, the insulin stimulation is abolished by amiloride, and insulin enhances the initial rate of $^{22}\text{Na}^+$ uptake.²¹ The addition of fresh serum or a variety of growth factors, including insulin, to quiescent cells in culture causes a rapid and striking increase in the activity of the Na/K pump, which was shown to be secondary to an increase in sodium influx and accumulation.^{8,13,14} Finally, insulin stimulates $^{86}\text{Rb}^+$ uptake in isolated rat adipocytes;²² however, in these cells insulin did not stimulate $^{22}\text{Na}^+$ uptake.^{22,23}

We have reported previously that incubation of HTC cells with insulin causes a two- to threefold induction of tyrosine aminotransferase and five- to tenfold increase in the initial rate of uptake of 2-aminoisobutyric acid,¹⁻⁴ but, that after 24-h incubation with insulin, the cells become completely unresponsive to the effects of insulin on these two phenomena.^{4,5} In contrast, similar incubation with insulin does not

cause complete unresponsiveness to the stimulation of ouabain-sensitive rubidium uptake by insulin. Although there is some diminution in the magnitude of stimulation of potassium influx by insulin under these conditions, it is clear from Figure 10 that the maximal rate of insulin-stimulated potassium influx is the same. Thus, most of the stimulation of potassium influx is retained after 24 h incubation with insulin.

The insulin stimulation of ion transport differs in several ways from other insulin effects in HTC cells. The induction of tyrosine aminotransferase and the stimulation of amino acid transport take longer to reach their maximal stimulation (4 and 2 h, respectively)⁴ than does the maximal stimulation of rubidium uptake (approximately 1 h). Furthermore, both of the former require concomitant protein synthesis and are blocked by cycloheximide,^{1,3} whereas the early stimulation of pump activity is not. The mechanism by which insulin induces tyrosine aminotransferase activity has been shown to be a selective slowing in the rate of degradation of the enzyme;² the mechanism of the stimulation of amino acid transport remains unknown.

The insulin induction of unresponsiveness to further insulin action involves postbinding regulatory steps. Although insulin downregulates the number of insulin receptors in HTC cells, the regulation of responsiveness (as measured by transaminase induction and amino acid transport stimulation) can be dissociated from this receptor regulation, suggesting that it involves postbinding steps in hormone action.⁵ The fact that insulin does not cause desensitization to the insulin activation of the Na/K pump suggests that the site of regulation of insulin responsiveness must be distal to, or separate from, those events causing stimulation of pump activity.

The mechanisms by which the binding of insulin to its receptor triggers the cellular responses to this hormone are unknown. Insulin very rapidly stimulates the phosphorylation of the β -subunit of its own receptor, an effect mediated by a tyrosine-specific protein kinase activity intrinsic to the receptor itself.²⁴⁻²⁶ The role of this phenomenon in mediating insulin's biologic actions, however, remains to be established. Incubation of isolated plasma membrane preparations with insulin causes the release of one or more low-molecular-weight peptides that can mediate the activation or inactivation of several insulin-sensitive enzymes by modulating their state of phosphorylation.²⁷⁻²⁹ The possible role of these mediators in the insulin stimulation of ion fluxes, or the insulin stimulation of amino acid transport and enzyme induction, has not been tested. It is noteworthy that insulin has been reported to stimulate $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in isolated membrane fragments.³⁰ Furthermore, it has been suggested that insulin binding to its receptor might induce localized lipid phase transitions in the membrane,^{31,32} which could then affect the activity of Na/K ATPase.³³ Thus, it is possible that the insulin stimulation of ion fluxes is mediated by membrane-associated biochemical events quite proximal to the hormone-receptor interaction.

The possible role of insulin-stimulated tyrosine kinase activity, of mediator production, and of alteration in membrane phospholipid organization in the insulin induction of unresponsiveness is an area of current investigation. Comparison of the role of these events in the insulin regulation of rubidium transport with that of transaminase induction and amino acid

transport may provide a useful approach to investigating the mechanisms of postbinding regulation of hormone responsiveness.

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