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INSULIN STIMULATION OF AMINO ACID TRANSPORT IN ISOLATED RAT HEPATOCYTES IS INDEPENDENT OF HORMONE INTERNALIZATION

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SUMMARY

Isolated rat hepatocytes were used to investigate the relationship between the effect of insulin on amino acid transport and hormone internalization. As previously observed with fibroblastic cells, 10 mM methylamine inhibited the clustering and internalization of the hormone-receptor complex in hepatocytes. Direct measurement of ^{125}I-insulin binding indicated that methylamine did not decrease the binding capacity of the cells. When used at concentrations that did not affect the basal rate of α-amino-isobutyric acid transport, methylamine did not cause a specific decrease in the stimulation by insulin. The data indicate that the internalization of insulin is not required for the expression of its biological effect on amino acid transport.

INTRODUCTION

Insulin is able to stimulate amino acid transport in freshly isolated hepatocytes from normal rats (1). The effect of insulin is detectable after 30 to 45 min incubation, but maximal stimulation of transport requires 2 to 3 hr to be expressed. Insulin effects are mediated through a first step involving direct interaction of the hormone with its receptor sites on the plasma membrane. The subsequent events which lead to the action of the hormone are poorly understood. It has recently been reported from *in vitro* (2, 3, 4) and *in vivo* (5) studies that insulin is internalized into hepatocytes. Similarly, fibroblastic cells in culture have been shown to internalize insulin through a mechanism which involved the aggregation of the occupied receptors (6). The processes of aggregation and internalization are specifically inhibited by various primary amines (7).

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In the present study we used freshly isolated hepatocytes to investigate the relationship between insulin stimulation of amino acid transport and internalization of the hormone. Our data indicate that internalization of insulin in the hepatocyte is not required for its biological effect on α-aminoisobutyric acid (AIB) transport.

MATERIALS AND METHODS

Hepatocytes were isolated from 5- to 6-week-old male rats fed ad libitum using a procedure previously described (8). The cell suspensions satisfied the criteria previously established to assess the preservation of cell structure and functions (9). For transport experiments, hepatocytes at 1×10^6 cells/ml were preincubated in the presence or absence of hormone and methylamine for 150 min in Krebs Ringer bicarbonate buffer (pH 7.5), containing 1% (w/v) bovine serum albumin (Fraction V) at 37°C gassed with 95% O_2/5% CO_2. The cells were then sedimented by centrifugation (1 min at 1000 rpm) and resuspended in fresh medium without hormone and methylamine; amino acid transport was measured for 5 min by the procedure previously reported (9). The binding of insulin to hepatocytes was measured at 37°C after steady state was reached (30 min), using the procedure described elsewhere (1). When indicated, methylamine was added 15 min prior to the addition of the mixture of ^{125}I-insulin and unlabeled hormone to the cell suspension. In order to minimize hormone degradation (10), cells were preincubated for 30 min at 37°C, washed once and resuspended in fresh medium before being used for binding studies; moreover, the cell concentration was kept low (0.5×10^6/ml). Analysis of the effect of methylamine on the aggregation of insulin receptors was examined both with hepatocytes in suspension and with hepatocytes that had been allowed to stick and to spread out on a plastic tissue culture dish.

When examined in suspension, 10 nM rhodamine-insulin was added to the cells under the conditions used for the transport studies. After 20 min at 37°C, the cells were sedimented and resuspended 3 times in Dulbecco's phosphate buffered saline (0°C) containing the concentration of methylamine used for the incubation (0 or 10 mM). In order to attach cells to the substratum, freshly isolated hepatocytes were washed 3 times in Eagle's medium supplemented with fetal calf serum (10% v/v), containing glutamine (2 mM), streptomycin (50 µg/ml) and penicillin (50 IU/ml). Cells were then seeded in 35-mm diameter plastic culture dishes (2×10^5 cells/dish). They attached to the substratum within 30 to 45 min and cell spreading was complete enough after 4 hr to allow investigation of the receptor aggregation process. The procedure used to study the effect of methylamine on the aggregation of insulin receptors involved the use of a fluorescent rhodamine derivative of insulin and was identical to that used with fibroblastic cells (6, 11).

Collagenase (185 U/mg), cyclic 3':5'-AMP, α-aminoisobutyric acid (AIB) and methylamine (hydrochloride salt) were purchased from Sigma.
α-Amino-[1-^{14}C]isobutyric acid (specific activity = 59 mCi/mmol) was from New England Nuclear. ^{125}I-insulin was prepared at a specific activity of 200 to 250 µCi/µg by a modification of the chloramine-T-method (12). (Rhodamine-lactalbumin)-insulin was prepared by Meloy Laboratories using the method of Schechter et al. (13).

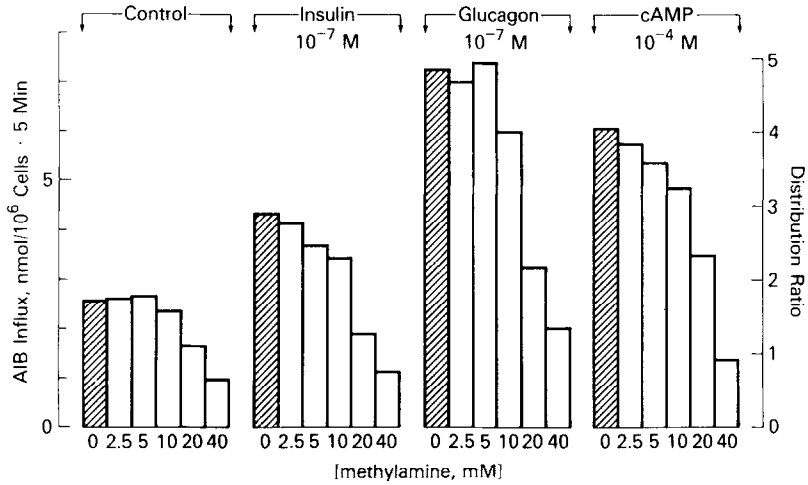

Figure 1. Effect of varying concentrations of methylamine on basal and hormone-stimulated AIB transport in isolated hepatocytes. Cells were first incubated for 150 min (see Materials and Methods) in the presence or absence of methylamine and hormone concentrations indicated. Transport of 0.1 mM ^{14}C-AIB was then measured for 5 min. The distribution ratio was calculated by dividing the intracellular AIB concentration by the extracellular concentration. Intracellular AIB concentration was calculated assuming an average water cell volume of 3 µl/10^6 hepatocytes. Each column represents the mean of triplicate determinations.

RESULTS

Fig. 1 shows the effect of varying concentrations of methylamine on basal and hormone-stimulated AIB transport. The initial rate of AIB transport (influx) in isolated hepatocytes was not altered when cells were incubated for 150 min in the presence of methylamine at concentrations ranging from 2.5 mM to 10 mM. In contrast, as indicated by the changes in the distribution ratio, the active transport of AIB was decreased by about 30% and 90% when 20 mM and 40 mM methylamine were used, respectively.

When used at 10^{-7} M, which represents the maximally effective concentration, insulin increased AIB influx by about 100%. The insulin effect was slightly inhibited by methylamine at 2.5 mM to 10 mM, concentrations which did not affect basal transport. In order to investigate the specificity of methylamine effect on insulin action, similar experiments were carried out with glucagon and cyclic 3':5'-AMP. Maximally effective doses of glucagon (10^{-7} M) and cyclic 3':5'-AMP (10^{-4} M) increased active AIB transport by

about 200% and 150%, respectively. When used at low concentrations (2.5 mM to 10 mM), methylamine also produced a small inhibitory effect on hormone stimulation of transport similar to the inhibition observed with insulin (Fig. 1). Exposure of hepatocytes to 20 mM methylamine virtually abolished the hormonal stimulation of transport. In the presence of 40 mM methylamine, the ability of hepatocytes to concentrate amino acids (i.e., active transport) was almost completely abolished, regardless of the presence of hormone. The effect of high methylamine concentration on AIB transport was accompanied by extensive swelling and vacuolization of the cell; this could account, at least partly, for the loss of active transport capacity.

Taking into account the foregoing observations, a concentration of 10 mM methylamine was selected to analyze the effect of methylamine on the dose-dependent stimulation of AIB transport by hormones (Fig. 2). The partial

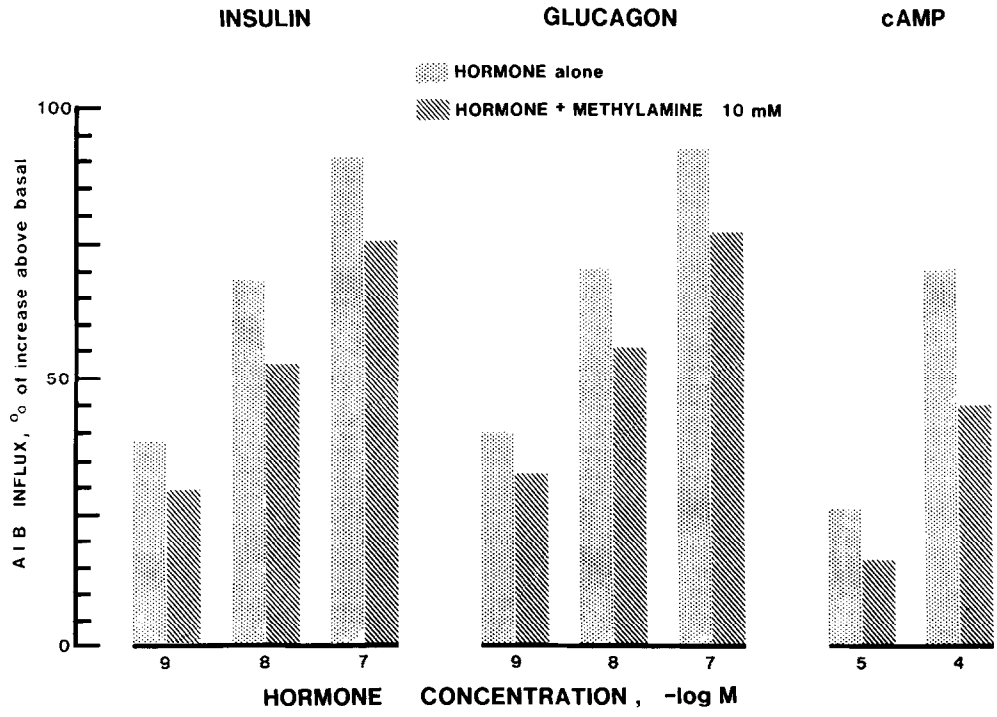

Figure 2. Effect of 10 mM methylamine on the dose-dependence relationship of insulin, glucagon and cyclic 3':5'-AMP effect on AIB transport. Hepatocytes were preincubated for 150 min in the presence and absence of methylamine 10 mM, with the various hormone concentrations indicated. Transport of 0.1 mM ^{14}C-AIB was then measured for 5 min. Each column represents the mean of triplicate determinations.

inhibition (~20%) by 10 mM methylamine of the stimulation of AIB transport by a maximally effective concentration (10^{-7} M) of insulin was also observed when submaximal concentrations (10^{-9} M and 10^{-8} M) were used. Similarly, 10 mM methylamine partially (20% to 40%) inhibited the action of glucagon and cyclic 3':5'-AMP on transport, regardless of the concentration of hormone used. It therefore appears that methylamine does not change the sensitivity of the cell toward hormones, but partially reduces the maximal effect on AIB transport in a nonspecific manner.

To test the possibility that the effect of methylamine on insulin stimulation of AIB transport could be mediated through modifications in the capacity of hepatocytes to bind insulin, we analyzed the effect of methylamine on the steady state level of insulin binding (Fig. 3). Methylamine at 10 mM increased the amount of hormone specifically bound by about 20% at all the concentrations tested; however, when 20 mM methylamine was used, minimal changes in the insulin-binding capacity of cells were observed. Accordingly, the methylamine inhibition of the effect of insulin on AIB transport cannot be accounted for by a decreased capacity of hepatocytes to bind the hormone.

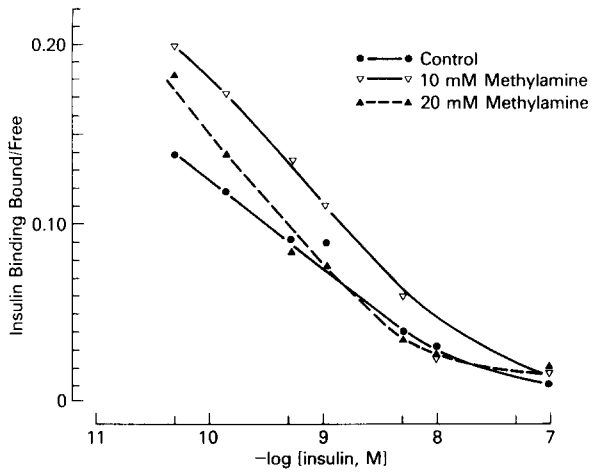

Figure 3. Effect of methylamine on the dose-dependence relationship of insulin binding. Incubation conditions are described in Materials and Methods. Each point represents the mean of triplicate determinations.

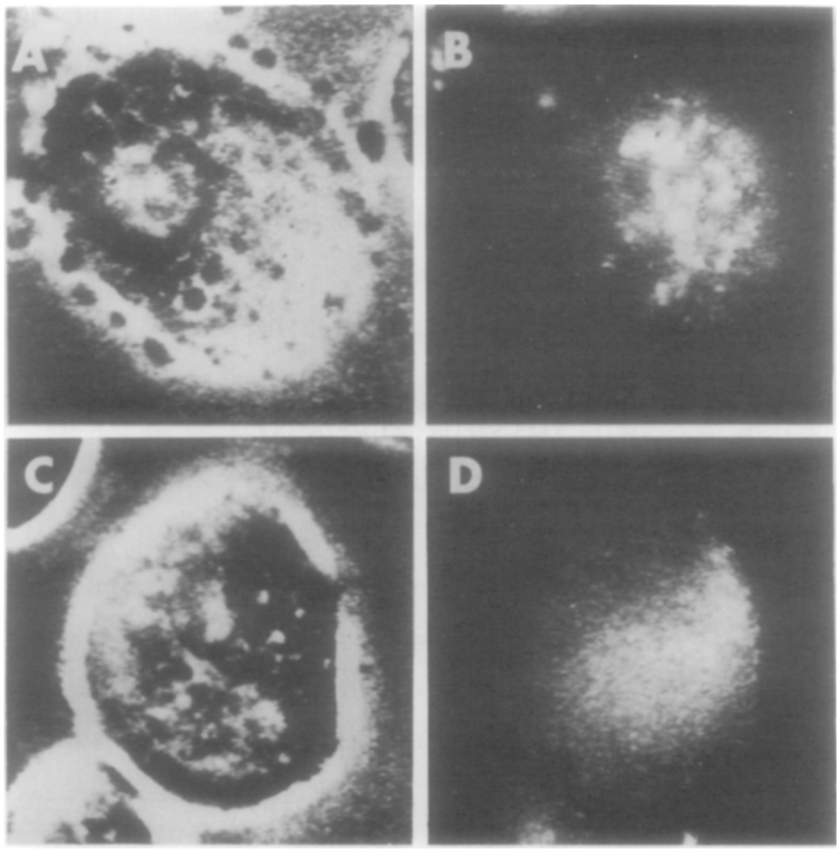

Figure 4. Effect of methylamine on the binding and internalization of rhodamine-insulin by attached hepatocytes. A and B: Rhodamine-insulin (10 nM) was added to the culture medium of hepatocytes which had attached to the substratum. After a 20 min incubation at 37°C, the cells were rinsed and fixed with 2% formaldehyde. C and D: Same as in A and B except methylamine (10 mM) was added to the culture medium 20 min before adding rhodamine-insulin. The cells were observed using a silicon intensifier target television camera, and pictures of the video monitor were taken. A and C: Rhodamine-insulin fluorescence; B and D: Phase contrast of the same cells as in A and C, respectively. Magnification x 1200.

Aggregation of receptors is an early event which takes place before internalization of the complex formed by the association of receptor and hormone molecules occurs (6, 11). As observed with fibroblastic cells (6), binding and internalization of fluorescent derivative of insulin can be visualized in monolayer culture of hepatocytes (Figs. 4A and 4B). The binding of the fluorescent derivative is specific, as it can be competed out by an excess of insulin. After 15 min at 37°C the fluorescent ligands were

distributed in a punctuate distribution on the hepatocyte, analogous to that described with fibroblastic cells (6). The pattern is characteristic of clustering and endocytosis of the receptor ligand complex. When 10 mM methylamine was added to the culture medium for 15 min before adding rhodamine-labeled insulin, a diffuse pattern of fluorescence was observed (Figs. 4C and 4D), indicating the receptor-mediated endocytosis was inhibited. Similar results were obtained with rhodamine-labeled α_2-macroglobulin (not shown). When rhodamine-insulin was added to suspended hepatocytes, it was rapidly internalized and was concentrated in a region near the nucleus (Figs. 5A

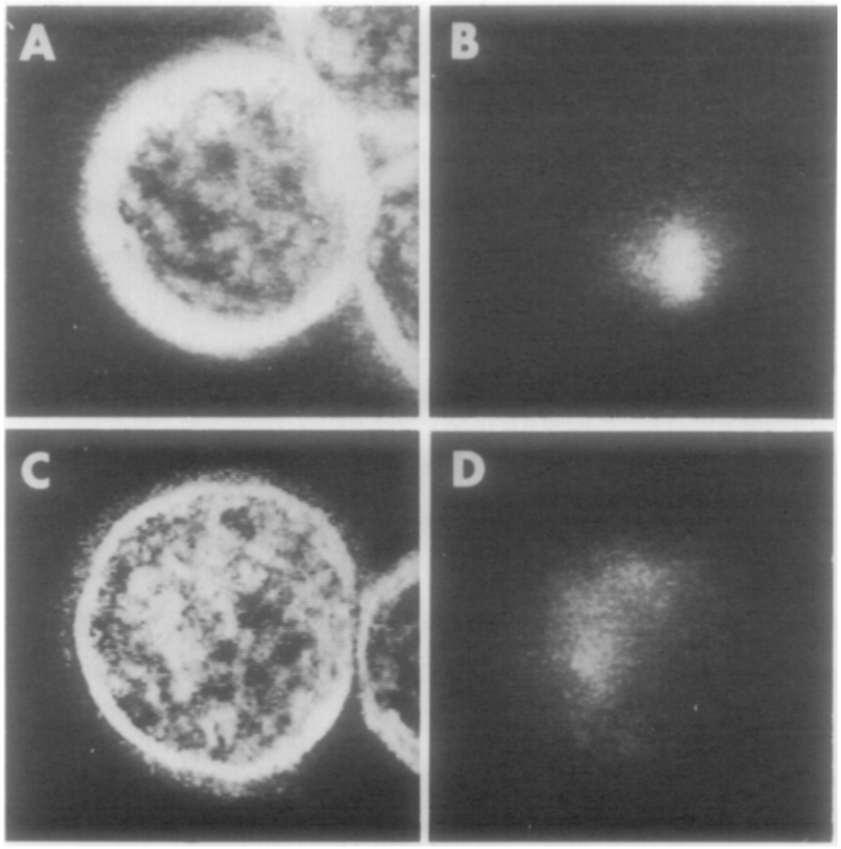

Figure 5. Effect of methylamine on the binding and internalization of rhodamine-insulin by suspended hepatocytes. A and B: Rhodamine-insulin (10 nM) was added to the incubation medium of suspended hepatocytes. After 20 min at 37°C, the cells were pelleted and rinsed (see Materials and Methods). C and D: Methylamine (10 mM) was added to the medium for 20 min before adding the rhodamine-insulin. Pictures of the video monitor were taken as in Fig. 4. A and C: Rhodamine-insulin fluorescence; B and D: Phase contrast of the cells shown in A and C, respectively. Magnification x 1900.

and 5B). In the presence of 10 mM methylamine, the fluorescence was more diffusely distributed. Some internalization and concentration were observed with methylamine present, but the extent of internalization was greatly reduced. This result indicates that in hepatocytes, as with fibroblasts, methylamine inhibits receptor-mediated endocytosis.

DISCUSSION

The question of whether various aspects of insulin action require internalization or whether interaction with cell surface receptors without internalization is sufficient has received a great deal of attention.

We have recently developed a method that allows direct visualization of the binding and internalization of insulin and other ligands on living cells (6, 11). With this method we found that various primary amines, including methylamine, inhibit in a reversible manner the aggregation and internalization of insulin, epidermal growth factor and α_2-macroglobulin (7). This finding prompted us to investigate whether or not the action of insulin is affected by methylamine.

One action of insulin that has been carefully studied is its ability to increase AIB uptake in rat hepatocytes (1). This action of insulin requires ongoing protein synthesis since it is blocked by cycloheximide (1).

Our results indicate that hepatocytes handle insulin like fibroblasts. The initial binding of insulin is to diffusely distributed receptors. Following this the hormone-receptor complexes aggregate and are internalized. The aggregation and internalization are blocked by methylamine. The binding of insulin to the cell is unaffected (Fig. 3).

When the effect of methylamine on insulin stimulation of AIB transport was studied, we found methylamine did not cause a specific inhibition of the action of insulin. Methylamine did have a nonspecific inhibitory effect which extended to cyclic AMP and glucagon. The effect of glucagon is mediated via cyclic AMP and does not require internalization. Cyclic AMP enters the cell by a nonsaturable mechanism distinct from that of insulin. Therefore,

we conclude that the action of insulin on AIB uptake does not require aggregation and internalization.

We have also been examining the effect of methylamine on the stimulation of DNA synthesis by epidermal growth factor. This action of EGF also is not blocked by methylamine. In fact, this action of EGF is potentiated by methylamine (Maxfield, in preparation). Thus, two complex actions of two different peptide hormones do not require either aggregation of hormones into coated pits or internalization. We speculate that internalization is required for hormone degradation and down-regulation of specific receptors (14). Experiments to evaluate these two mechanisms are now in progress.

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REFERENCES

1. LeCam, A., and Freychet, P. (1978) Diabetologia 15, 117-123.
2. Carpentier, J. L., Gorden, P., LeCam, A., Freychet, P., and Orci, L. (1977) Diabetologia 13, 386.
3. Gorden, P., Carpentier, J. L., Freychet, P., LeCam, A., and Orci, L. (1978) Science 200, 782-785.
4. Carpentier, J. L., Gorden, P., Barazzone, P., Freychet, P., LeCam, A., and Orci, L. (1979) Proc. Natl. Acad. Sci. USA, in press.
5. Bergeron, J. J. M., Sikstrom, R., Hand, A. R., and Posner, B. I. (1979) J. Cell Biol. 80, 427-443.
6. Schlessinger, J., Schechter, Y., Willingham, M. C., and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 2659-2663.
7. Maxfield, F. R., Willingham, M. C., Davies, P. J. A., Pastan, I. (1979) Nature 277, 661-663.
8. LeCam, A., Guillouzo, A., and Freychet, P. (1976) Exp. Cell Res. 98, 382-395.
9. LeCam, A., and Freychet, P. (1977) J. Biol. Chem. 252, 148-156.
10. Terris, S., and Steiner, D. F. (1975) J. Biol. Chem. 250, 8389-8398.
11. Maxfield, F. R., Schlessinger, J., Schechter, Y., Pastan, I., and Willingham, M. C. (1978) Cell 14, 805-810.
12. Freychet, P. (1976) Methods in Receptor Research, Part II, pp. 385-428, Blecher, M. ed. Marcel Dekker, Inc., New York.
13. Schechter, Y., Schlessinger, J., Jacobs, S., Chang, R. D., and Cuatrecasas, P. (1978) Proc. Natl. Acad. Sci. USA 75, 2135-2139.
14. Gavin, J. R. III, Roth, J., Neville, D. M. Jr., De Meyts, P., and Buell, D. N. (1974) Proc. Natl. Acad. Sci. USA 71, 84-88.