Effects of monensin on insulin interactions with isolated hepatocytes

Evidence for inhibition of receptor recycling and insulin degradation

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Recent evidence suggests that, during endocytosis, receptors for many polypeptide ligands are spared degradation and are recycled to the plasma membrane for re-utilization. The univalent ionophore monensin was shown to inhibit membrane recycling. We therefore examined its effects on insulin interactions with isolated rat hepatocytes to characterize further receptor endocytosis and recycling in these cells. At 10 °C, in the absence of endocytosis, no change in insulin binding was observed. However, at 37 °C a concentration-dependent decrease in ^{125}I-insulin binding was seen in the presence of insulin; this reached a maximum of 60% at 1 nM-insulin. Competitive binding studies showed this to be due to a 50–60% decrease in cell-surface insulin-receptor concentration, although the total cellular receptor concentration remained unchanged, suggesting that monensin causes the intracellular sequestration of receptors. Time-course studies of the processing of 2.5 nM-insulin showed that monensin produced a 50–60% decrease in surface binding, accompanied by a similar decrease in internalization and total inhibition of insulin degradation. When hepatocytes with ^{125}I-insulin prebound to their surface receptors at 10 °C were warmed to 37 °C, monensin had no effect on internalization, but caused marked impairment of intracellular insulin degradation. It is concluded that monensin inhibits receptor recycling and cellular insulin degradation.

INTRODUCTION

Many polypeptides which bind to cell-surface receptors are internalized by their target cells and subsequently degraded, probably in lysosomes (Gorden et al., 1980). Although in many cases receptors enter the cell with their ligands, the further processing of the receptor remains unclear. It has been suggested for certain ligands that the receptor may escape the fate of the ligand and be recycled back to the plasma membrane for re-utilization in the endocytotic process. This has been demonstrated for fibroblast low-density-lipoprotein receptors (Goldstein et al., 1976), asialoglycoprotein receptors in hepatocytes (Steer & Ashwell, 1980), mannose-conjugate glycoprotein receptors in macrophages (Stahl et al., 1980) and lysosomal-enzyme receptors in fibroblasts (Gonzalez Noriega et al., 1980). Other studies have shown that, in hepatocytes (Desbuquois et al., 1982; Fehlmann et al., 1982), adipocytes (Marshall et al., 1981) and 3T3-C2 fatty fibroblasts (Knutson et al., 1983), insulin and its receptor undergo a similar sequence of events during endocytosis.

The univalent cationic ionophore monensin has been shown to disrupt the transport of membrane vesicles from the Golgi apparatus to the plasma membrane in eukaryotic cells (Johnson & Schlessinger, 1980). In cells treated with monensin, secretion of soluble proteins is blocked (Tartakoff & Vassalli, 1978) and the transfer of membrane proteins from the Golgi to the plasma membrane is impaired (Strous & Lodish, 1980). Monensin has been shown to interrupt the recycling of fibroblast low-density-lipoprotein receptors (Basu et al., 1981). Thus, in the present study, we have examined the effects of monensin on the interaction of insulin with

isolated rat hepatocytes. We have been able to demonstrate that monensin inhibits the recycling of insulin receptors and we have further characterized the endocytotic process, using this drug.

MATERIALS AND METHODS

Animals

Male Wistar rats (180–200 g) maintained at constant temperature (22 °C) on a constant 12 h-light/12 h-dark cycle were used for all studies. Animals were allowed free access to standard laboratory chow and water.

Materials

Collagenase (P-L Biochemicals; lot no. 504-22) was purchased from Windsor Laboratories, Windsor, Berks., U.K. Bacitracin (71.5 units/μg), Hepes, Tricine and monensin were from Calbiochem–Behring, C-P Laboratories, Bishops Stortford, Herts., U.K. Aprotinin, phenylmethanesulphonyl fluoride, bovine serum albumin and human serum albumin (fraction V) were from Sigma (London) Chemical Co., Poole, Dorset, U.K.; Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglucuronyl) was from Pierce and Warriner, Chester, U.K. Na^{125}I was from The Radiochemical Centre, Amersham, Bucks., U.K. Crystalline pig monocomponent insulin (Actrapid) was generously given by Dr. L. Heding, Novo Research Institute, Bagsvaerd Allee, Copenhagen, Denmark. All other reagents were A.R. grade and were purchased from B.D.H., Poole, Dorset, U.K. Monensin was prepared as a 10 mM stock solution in ethanol, and equal volumes of ethanol were added to all controls.

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Hepatocyte isolation

Hepatocytes were isolated from fed rats by the method of Fehlmann et al. (1979). Cells isolated in this way were 93–95% viable, as judged by Trypan Blue exclusion, and viability was unaffected by experimental procedures. For all studies cells were suspended at a final concentration of 2.4×10^6/ml in Krebs–Ringer–Hepes buffer, pH 7.4 (Vinten et al., 1973), containing defatted bovine serum albumin (30 g/l; Chen, 1967), aprotinin (2000 kallikrein-inhibitory units/ml) and bacitracin (1 mg/ml). Cells were routinely preincubated for 40 min at 37 °C before all experimental procedures.

Insulin iodination

Pig monocomponent insulin was iodinated with Iodogen to a specific radioactivity of 180–200 μCi/μg by a modification of the method of Fraker & Speck (1978) and then purified by chromatography on a Sephadex G-50 column (0.9 cm × 60 cm) eluted with 200 mM-glycine buffer, pH 8.6, containing human serum albumin (2.5 g/l) and merthiolate (2.5 mg/ml). ^{125}I-insulin prepared in this manner has an affinity for rat liver plasma-membrane insulin receptors of $\geq 80\%$ that of [A14-mono[^{125}I]iodotyrosyl]insulin (J. Whittaker, unpublished work).

Insulin binding

Hepatocytes (1×10^6–2×10^6/ml) were incubated with ^{125}I-insulin (33 pM) and insulin (0–100 nM) in a final volume of 500 μl in 15 cm × 100 cm tubes gassed with O_2/CO_2 (19:1). Incubations were terminated by removing 400 μl samples of cells and placing them in 3.5 ml of ice-cold Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), in 12 cm × 75 cm conical centrifuge tubes. Cells were sedimented by centrifugation for 40 s at 2000 $g_{av.}$ and the supernatant was aspirated. The cells were washed once without resuspension with ice-cold Krebs–Ringer bicarbonate buffer and re-centrifuged. Non-specific binding was determined as radioactivity bound in the presence of 10 mM unlabelled insulin and represented < 3% of binding of tracer at 37 °C and 7–10% at 10 °C. All incubations were performed in triplicate.

Insulin internalization

Internalization of insulin by hepatocytes was determined by a modification (Whittaker et al., 1983) of the method developed by Haigler et al. (1980) for the measurement of endocytosis of epidermal growth factor by fibroblasts. Briefly, 400 μl of cells, incubated as for binding studies, were placed in 3.5 ml of 0.3 M-acetic acid in 0.6 M-NaCl at 4 °C for 5 min and then separated as for binding studies. When cells treated in this manner after a 120 min incubation at 37 °C with ^{125}I-insulin (200 pM) were extracted in 6 M-urea/3 M-acetic acid/0.1% Triton X-100 and chromatographed on Sephadex G-50, more than 95% of the applied radioactivity was eluted in the position of ^{125}I-insulin. Correction for non-specific internalization was made in the same manner as for binding studies. Surface-bound insulin was thus determined as the difference between total cell-associated insulin and internalized insulin measured in parallel.
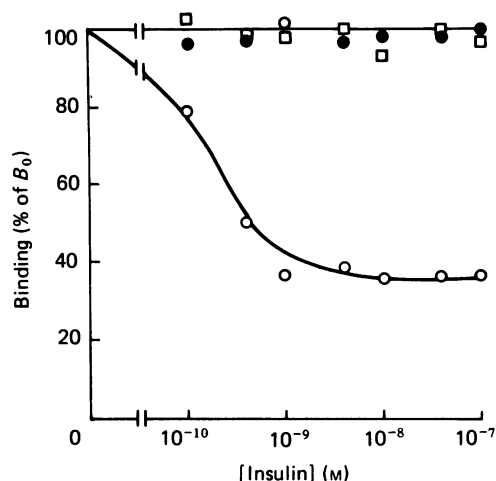

Fig. 1. Effects of monensin and insulin on the surface binding of insulin by hepatocytes

After a 15 min preincubation at 37 °C in the presence of monensin (25 μM), hepatocytes were incubated for 90 min at 37 °C ($\bigcirc$) or 10 °C ($\square$) with the indicated concentrations of insulin and monensin (25 μM). Control cells ($\bullet$) were preincubated for 15 min at 37 °C without monensin and then incubated for a further 90 min at 37 °C in monensin-free buffer with the indicated concentration of insulin. The cells were then washed with phosphate-buffered saline, pH 6.5, to remove bound unlabelled insulin as described in the text, and specific binding of ^{125}I-insulin (33 pM) was determined after a 90 min incubation at 10 °C. Results are expressed as % of B_0 where B_0 is the ^{125}I-insulin bound by cells that had been preincubated in the absence of unlabelled insulin. In cells incubated for 90 min at 37 °C with 25 μM-monensin, B_0 was not significantly different from that in control cells ($5.0 \pm 0.19\%/10^9$ cells per 1 for monensin-treated cells and $4.98 \pm 0.15\%/10^9$ cells per 1 for control cells; means $\pm$ s.e.m. of triplicate samples). These data represent one of three representative experiments.

Insulin binding to solubilized hepatocytes

Hepatocytes (4×10^7–8×10^7) were sedimented by centrifugation at 100 $g_{av.}$ and the pellet was homogenized in 5 ml of 0.2 M-NaCl/50 mM-Hepes, pH 7.5, containing 1 mM-N-ethylmaleimide, 2 mM-phenylmethanesulphonyl fluoride, aprotinin (2000 kallikrein-inhibitory units/ml) and bacitracin (4 mg/ml). Triton X-100 was added to a final concentration of 1% (v/v) and the homogenate was extracted for 60 min at 4 °C. The resulting suspension was centrifuged at 100000 $g_{av.}$ for 60 min at 4 °C. The supernatant contained 90–95% of the binding activity of a four-times-freeze–thawed homogenate.

For insulin-binding studies, 300 μl of a 1:10 dilution of the 100000 g supernatant in Krebs–Ringer–Hepes buffer containing defatted bovine serum albumin (30 g/l), aprotinin (2000 kallikrein-inhibitory units/ml) and bacitracin (1 mg/ml) were incubated with ^{125}I-insulin (33 pM) and insulin (0–100 nM) in a final volume of 0.5 ml for 90 min at 10 °C. Separation of bound and free insulin was achieved by poly(ethylene glycol) precipitation as described by Harrison & Itin (1980). All incubations were performed in duplicate.
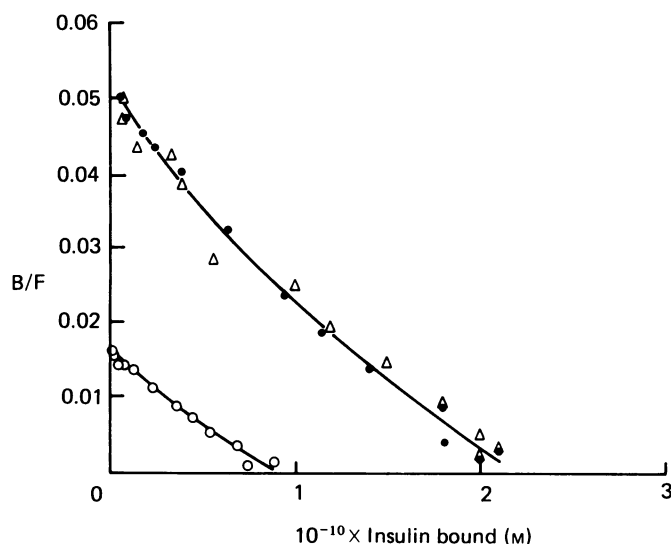
Fig. 2. Effect of monensin and insulin on cell-surface insulin-receptor concentration of isolated hepatocytes

Hepatocytes were preincubated with or without insulin (2.5 nM) in the presence or absence of monensin (25 μM) for 45 min at 37 °C. Cells were washed with phosphate-buffered saline, pH 6.5, to remove bound insulin, and competitive binding studies with ^{125}I-insulin were performed as described in the text. For monensin-treated cells, all procedures were carried out in the presence of 25 μM-monensin. The results of one of three representative experiments are represented as Scatchard (1949) plots. B/F is the ratio of bound to free ^{125}I-insulin, and insulin bound is the insulin bound per 10^9 cells/l. ●, Control; △, 2.5 nM-insulin; ○, 2.5 nM-insulin and 25 μM-monensin.

Insulin degradation

This was determined by trichloroacetic acid precipitation by the method of Freychet *et al.* (1972).

Hepatocyte washing

In order to remove receptor-bound insulin from hepatocytes for the measurement of ^{125}I-insulin binding after preincubation with unlabelled insulin, cells were sedimented by centrifugation for 3 min at 100 $g_{av.}$ and then resuspended in 40 ml of phosphate-buffered saline, pH 6.5 (Dulbecco, 1954), containing bovine serum albumin (10 g/l) at room temperature and immediately re-centrifuged. This was repeated twice. The cells were finally resuspended in 40 ml of the same medium for 20 min at room temperature, re-centrifuged and re-suspended in binding buffer for binding studies. This procedure had no effect on the viability of the cells, specific tracer binding or non-specific binding of insulin.

RESULTS

To demonstrate the effects of monensin on insulin receptors, hepatocytes were incubated for 90 min in the absence or presence of monensin (25 μM) with various concentrations of insulin at 10 °C or 37 °C. After extensive washing to remove receptor-bound unlabelled insulin, binding of ^{125}I-insulin (33 pM) was determined at 10 °C, to obtain a measure of cell-surface insulin binding; endocytosis of insulin is minimal at this temperature

(Whittaker *et al.*, 1983). Hepatocytes preincubated at 37 °C with monensin and insulin showed a decrease in ^{125}I-insulin binding which was dependent on the concentration of insulin present during the preincubation (Fig. 1). A maximal decrease of 62% was observed at an insulin concentration of 1–4 nM. In contrast, there was no change in ^{125}I-insulin binding in control cells or hepatocytes preincubated with monensin and insulin at 10 °C. When cells were incubated with monensin alone, no change in insulin binding was observed (see legend to Fig. 1).

In order to determine the mechanisms of the monensin- and insulin-induced decrease in ^{125}I-insulin binding, competitive binding studies at 10 °C were performed after preincubation with insulin at 37 °C in the presence or
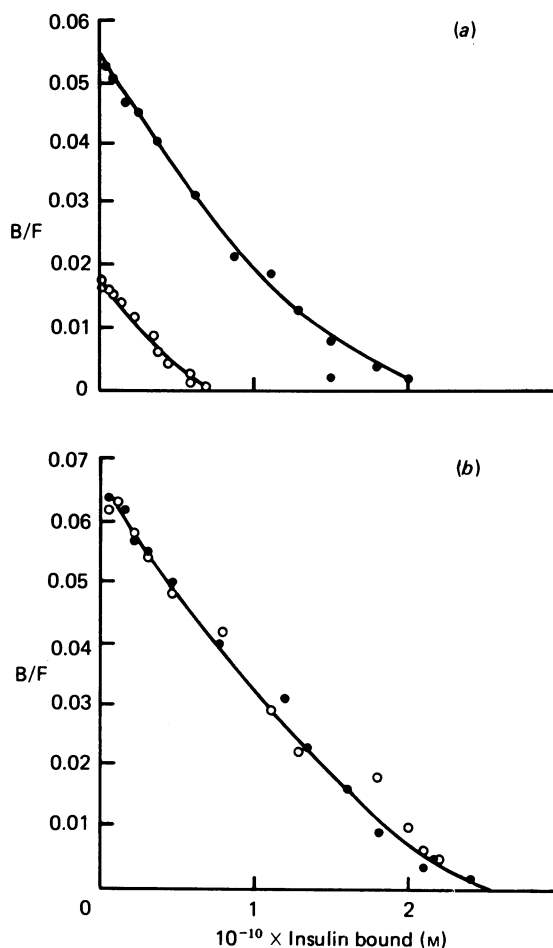

Fig. 3. Effect of monensin and insulin on surface and total cellular insulin-receptor concentration of hepatocytes

Hepatocytes were preincubated with 25 nM-insulin in the presence (○) or absence (●) of monensin (25 μM) for 45 min at 37 °C. Cells were washed with phosphate-buffered saline, pH 6.5, in the presence or absence of monensin (25 μM) as described in the text. (*a*) Scatchard plot of surface binding; binding of ^{125}I-insulin at the indicated concentrations of unlabelled insulin was determined after a 90 min incubation at 10 °C. (*b*) Scatchard plot of total binding capacity. Cells were solubilized and ^{125}I-insulin binding to solubilized receptors was determined as described in the text. These data represent the results of one of three representative experiments.
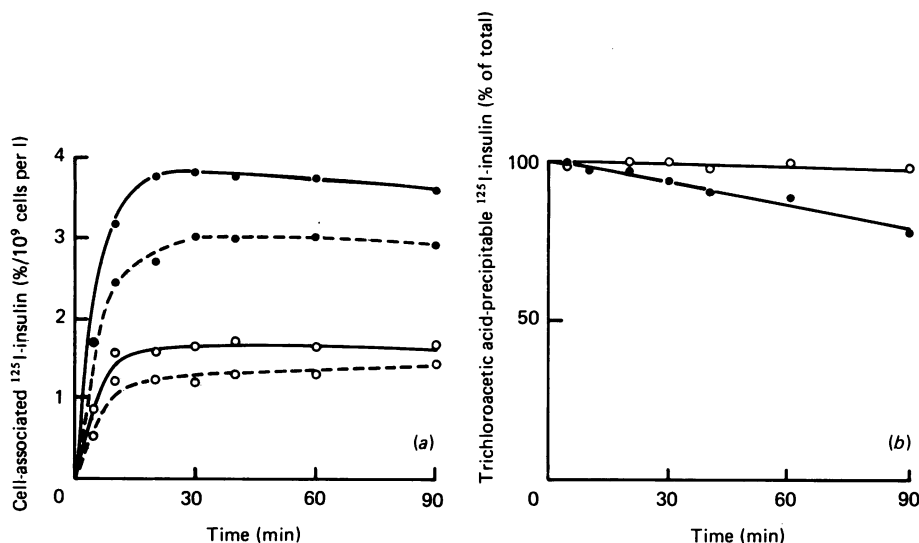
Fig. 4. Effects of monensin on the time course of binding, internalization and degradation of insulin

(a) Binding and internalization of insulin. After a 15 min incubation in the presence (○) or absence (●) of 25 μM-monensin at 37 °C, the binding (———) and internalization (————) of 2.2 nM-insulin and 0.2 nM-^{125}I-insulin was determined as described in the text. (b) Degradation. Degradation in the above experiment were determined by trichloroacetic acid precipitation. See the text for further details.

absence of monensin. Fig. 2 shows these results expressed as Scatchard (1949) plots. The abscissa intercepts indicate that, although there is a 60% decrease in receptor concentration in monensin-treated cells, insulin alone had no effect. To determine whether this was due to a redistribution of receptors or a decrease in total cellular receptors, ^{125}I-insulin binding to Triton X-100-solubilized hepatocytes was measured at 10 °C after the cells had been preincubated with insulin at 37 °C in the presence or absence of monensin. Surface receptor concentration decreased by 60% in the presence of monensin (25 μM), but there was no significant difference in the cell binding capacity determined in the solubilized cells (Figs. 3a and 3b).

Studies of the cellular uptake and degradation of insulin were performed to determine the effects of monensin on these processes. The time course of the binding, internalization and degradation of insulin (2.2 nM) by hepatocytes was determined at 37 °C in the absence or presence of monensin (25 μM). In monensin-treated cells, both total cell-associated ^{125}I-insulin and internalized ^{125}I-insulin were decreased by 50–60% at all time points studied (Fig. 4a). The effects were readily apparent as early as 5 min. Surface-bound radioactivity, derived from the difference between total and internalized radioactivity, was decreased to a similar extent (60%) at all time points in monensin-treated cells (results not shown). In addition, monensin completely inhibited degradation of ^{125}I-insulin, as measured by the decrease in trichloroacetic acid-precipitable radioactivity in the medium.

As it seemed unlikely that the inhibition of insulin degradation observed in the presence of monensin could be accounted for by inhibition of insulin uptake alone, the processing of ^{125}I-insulin pre-bound to receptors was studied in the presence or absence of monensin. In control cells (Fig. 5a) there is a rapid transfer of insulin from the cell surface to the intracellular compartment, reaching a

maximum at 10 min. At later time points the intracellular radioactivity declines. This is coincident with the appearance of trichloroacetic acid-soluble radioactivity in the medium; the pattern of decrease in intracellular radioactivity is a mirror image of the appearance of acid-soluble radioactivity. In the presence of monensin (Fig. 5b), clearance of insulin from the cell surface is virtually identical with that seen in the absence of monensin. In contrast, however, the onset of the decline in intracellular radioactivity is markedly retarded. Similarly the appearance of acid-soluble radioactivity is decreased. Thus it must be concluded that the major effect of monensin on the processing of pre-bound insulin is to impair its degradation.

DISCUSSION

We have demonstrated that in the presence of insulin monensin decreases the surface binding of insulin by isolated hepatocytes. This decrease is dependent on the integrity of endocytosis and varies with insulin concentration. It results from a decrease in cell-surface insulin-receptor concentration, although total cell receptor concentration remains unchanged. This suggests that the receptor is internalized with insulin and that monensin blocks replacement of receptors in the cell membrane.

Jacobs et al. (1983) have demonstrated that monensin inhibits the post-translational processing of insulin receptors in IM-9 lymphocytes. However, it is unlikely that this is responsible for the decrease in receptor concentration that we have observed, as the half-time for receptor biosynthesis is 9–12 h in IM-9 lymphocytes (Kasuga et al., 1981) and 3T3 L1 fatty fibroblasts (Reed & Lane, 1980), and it has been shown that the kinetics of receptor biosynthesis in hepatocytes are very similar (Van Obberghen & LeCam, 1981; Krupp & Lane, 1981). Inhibition of replacement from an intracellular pool can be excluded, as the intracellular pool of insulin receptors
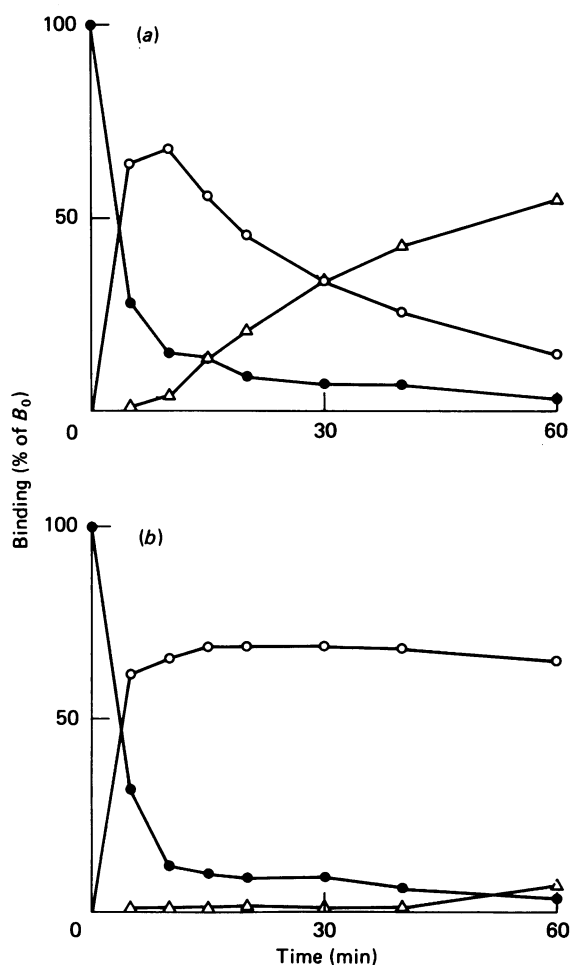
Fig. 5. Effect of monensin on processing of receptor-bound insulin by hepatocytes

After a 15 min preincubation in the presence or absence of monensin (25 mM), hepatocytes (2 × 10⁶/ml) were cooled to 10 °C and then incubated with 0.2 nM-insulin for 90 min at 10 °C. Cells were then washed four times with ice-cold Krebs–Ringer bicarbonate buffer. The cells were then resuspended in incubation medium at 37 °C in the presence or absence of monensin (25 μM). Surface-bound (O), internalized (●) and trichloroacetic acid-soluble medium radioactivity (△) were determined as indicated in the text. Results are expressed as % of B_0, where B_0 is the total cell-bound radioactivity immediately on resuspension of the cells. Each point represents the mean of duplicate determinations, except for B_0, which was determined in triplicate. (a) Processing of insulin by control cells; $B_0 = 7114 \pm 120$ c.p.m. (mean $\pm$S.E.M.). (b) Processing of insulin by monensin-treated cells; $B_0 = 7230 \pm 180$ c.p.m. (mean $\pm$S.E.M.).

represents only 25% of the cell-surface binding capacity, whereas the decrease in surface receptor concentration observed in the presence of monensin is 60% (Figs 3a and 3b), i.e. replacement of receptors from the intracellular pool would be inadequate to maintain receptor concentrations under these circumstances. Thus it appears that monensin inhibits receptor recycling.

As it was not possible to demonstrate any effect of monensin on hepatocyte insulin receptors in the absence of insulin, it would appear that these receptors are not

either internalized or recycled when unoccupied by insulin, or at least these processes occur too slowly to be detectable by our methods. This is in marked contrast with low-density-lipoprotein receptors (Basu et al., 1981), but agrees with the findings of Marshall et al. (1981) in adipocytes and Knutson et al. (1983) in 3T3 C fatty fibroblasts. This pattern of internalization is energetically more efficient for the cell and may be facilitated by the fact that the insulin receptor is also a metabolic signalling system, whereas the low-density-lipoprotein receptor appears to be only a transport protein. Thus the conformational changes involved in signal generation could initiate the internalization of the insulin-receptor complex.

The rate of recycling appears to be rapid. Our studies of the effects of monensin on the time course of the uptake of insulin (Figs. 5a and 5b) show that maximal decrease (50–60%) in surface insulin-receptor concentration takes place within 5–10 min of the addition of insulin to the cells. This is in marked contradiction to the finding of a recycling time of 6 h by Fehlmann et al. (1982), who used photoaffinity labelling of the receptor with ¹²⁵I-insulin and electron-microscope autoradiography to study recycling in isolated hepatocytes. It is, however, possible that these experimental procedures, i.e. u.v. irradiation and covalent coupling of insulin to the receptor, may have produced perturbations of the pathways of endocytosis and recycling that may account for this discrepancy. For example, it has been suggested that failure of dissociation of the ligand may impair recycling (Gonzalez Noriega et al. (1980). The rate of receptor recycling that we have observed is more consistent with that reported by Marshall et al. (1981) in adipocytes and Knutson et al. (1983) in 3T3 C fatty fibroblasts.

Only 60% of hepatocyte insulin receptors appear to be monensin-sensitive (Fig. 1). Although both monensin-sensitive and monensin-insensitive receptors appear to be fully functional with regard to insulin binding, our studies of the effect of monensin on insulin uptake by hepatocytes (Figs 5a and 5b) suggest that the latter class of receptors are not capable of internalization; the total amount of insulin (27 pmol) internalized in the presence of monensin represents only 14% of the total surface binding capacity of the hepatocytes (200 pmol; see Figs. 2 and 3a).

These experiments also suggest that occupancy of one binding site by insulin may lead to the internalization of more than the one occupied binding site. As noted above, only 27 pmol of ¹²⁵I-insulin was taken up by monensin-treated hepatocytes (Fig. 4a). In contrast, surface binding fell by 60%, i.e. internalization of 27 pmol of insulin was accompanied by the internalization of 120 pmol of receptor. Therefore, endocytosis of one occupied binding site must have been accompanied by the endocytosis of at least four empty sites.

The existence of two sub-populations of insulin receptors in hepatocytes and the finding that occupancy of one binding site leads to the internalization of multiple binding sites may be related to the molecular structure of the insulin receptor in the cell membrane. Structural studies of the insulin receptor suggest that it may exist in multiple oligomeric forms (Crettaz & Kahn, 1984), the simplest a heterodimer ($\alpha\beta$) of M_r 210000, and more complex thiol-linked oligomeric structures ranging in M_r up to 520000. Furthermore, these authors have shown that only the higher-M_r species of receptor are subject to

down-regulation, and hence the heterodimeric forms may therefore represent the monensin-resistant form of receptors. It would also be expected that occupancy of one binding site in a multi-subunit thiol-linked oligomer would lead to endocytosis of all the binding sites of the oligomer.

Monensin is thought to impair the orderly transfer of vesicles from the Golgi apparatus to the plasma membrane (Tartakoff & Vassalli, 1978). However, this does not necessarily imply that insulin receptors are recycled via the Golgi. It is evident from our studies that monensin also inhibits intracellular degradation of insulin. This is thought to be a lysosomal process (Terris et al., 1979), and the most likely mechanism of this action of monensin would be to increase intralysosomal pH. This has been reported for the univalent ionophore nigericin and also for chloroquine and NH_4Cl (Ohkuma & Poole, 1978). It is noteworthy that the latter substances also decrease insulin binding to hepatocytes (Whittaker et al., 1981) and that they have been reported to inhibit recycling of insulin receptors in adipocytes (Marshall et al., 1981), asialoglycoprotein receptors (Tolleshaug & Berg, 1979), lysosomal enzyme receptors (Gonzalez-Noriega et al., 1980) and mannose glycoconjugate receptors (Tietze et al., 1980). It therefore seems likely that maintenance of pH gradients across the membranes of intracellular vacuolar systems is essential to the integrity of the recycling process and that this process occurs via an endocytotic compartment which maintains a pH gradient.

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