Biochem. J. (1982) **206**, 295–299
Printed in Great Britain

Evidence for a direct effect of bacitracin on cell-mediated insulin degradation in isolated hepatocytes

Sandra M. JUUL and Richard H. JONES
Department of Medicine, St. Thomas's Hospital Medical School, Lambeth Palace Road, London SE1 7EH, U.K.

(*Received 23 February 1982/Accepted 27 April 1982*)

In freshly isolated hepatocytes, in which extracellular degradation of insulin was very low, the degradation velocity was first-order with respect to the amount of insulin bound at steady state. The addition of bacitracin decreased the degradation velocity considerably, so that a higher proportion of cell-associated radioactivity remained intact. The results demonstrate that bacitracin affects the mechanism of insulin processing by intact hepatocytes.

Insulin binding and degradation have previously been reported to be independent processes in preparations of isolated adipocytes, hepatocytes and plasma membranes (Freychet *et al.*, 1972; Gammeltoft & Gliemann, 1973; Kahn *et al.*, 1974; Olefsky *et al.*, 1975; Hammond & Jarrett, 1975; Gliemann & Sonne, 1978). In contrast, it has been reported (Terris & Steiner, 1975) that, with intact hepatocytes, insulin degradation velocity is directly proportional to total insulin bound at steady-state, a finding supported by subsequent studies using primary cultures of hepatocytes (Duckworth *et al.*, 1981; Caro & Armatruda, 1980). In many preparations of isolated hepatocytes, however, degradation of insulin occurs in the medium as well as in relation to receptor binding and, in cell preparations that leak degrading activity, the former is likely to be a large proportion of the total (Gammeltoft *et al.*, 1978). Studies of the relationship between receptor binding and degradation of insulin in preparations of isolated hepatocytes are often complicated by the occurrence of considerable extracellular degrading activity. Consequently, bacitracin, an inhibitor of certain proteinases (Makinen, 1972), has been used, at high concentrations (0.6–1.2mM), to inhibit extracellular degradation in order to study the internalization and processing of insulin by hepatocytes (Carpentier *et al.*, 1979). The leakage of degrading activity from a cell preparation implies a lack of integrity of the plasma membrane and, in addition, evidence that bacitracin has no direct effect on cells is lacking.

Careful attention to experimental detail has resulted in preparations of hepatocytes which do not leak large amounts of degrading activity, and with these cells we have been able to reassess the relationship between insulin binding and degradation and to examine the direct effect of high concentrations of bacitracin on these processes.

Materials and methods

Silastic tubing was from Dow Corning Corp., Midland, MI, U.S.A. Eagle's minimal essential medium (109G) was obtained from Gibco Bio-Cult, Glasgow, Scotland, U.K. Collagenase was from Boehringer Mannheim, Lewes, Sussex, U.K. Bovine serum albumin (essentially fatty acid free) and bacitracin were from Sigma Chemical Co., Poole, Dorset, U.K. Bacitracin was also obtained from Aldrich Chemicals, Gillingham, Dorset, U.K., and from Dumex Ltd., Copenhagen, Denmark (a gift from Dr. S. Gammeltoft). Bovine insulin (free of desamido form) was provided by Dr. D. Saunders of the Deutsches Wollforschunginstitut, Aachen, Germany. Tracers used were [A_{14}-^{125}I]monoiodoinsulin (pig and human), prepared with specific radioactivities of 324 and 359μCi/μg respectively, kindly provided by Eli Lilly, Indianapolis, IN, U.S.A. Monoiodoinsulin was also prepared from desamido-free bovine insulin by a chloramine-T method (Greenwood *et al.*, 1963) and fractionated by polyacrylamide-gel electrophoresis (Linde *et al.*, 1980).

Preparation of cells

Male Corworth–Sprague–Europe rats (150–200g), fed *ad libitum*, were anaesthetized with sodium pentobarbitone. The cells were isolated by a modification of the method of Seglen (1973), with Hanks bicarbonate buffer, pH 7.4 (minus Mg^{2+} and Ca^{2+}), as perfusion medium. The perfusion medium was maintained at 37°C and constantly gassed with O_2/CO_2 (19:1) by flow through a 'lung' consisting

0306-3283/82/080295-05$01.50/1

of coiled silastic tubing (Berry *et al.*, 1974). After recycling the perfusate for 10 min, the medium was replaced with the same buffer containing 4 mM-$CaCl_2$ and collagenase (0.5 mg/ml), and perfusion was continued for a further 5 min. The liver was excised and placed in 30 ml of ice-cold Eagle's minimal essential medium supplemented as described by Terris & Steiner (1975) and containing 1% bovine serum albumin (essentially fatty acid free). The cells were gently teased out, filtered through nylon mesh and incubated at 37°C for 30 min with constant gassing. Thereafter the cells were cooled on ice, filtered and centrifuged at 100 *g* for 2 min at 4°C. They were then washed three times with ice-cold Hanks bicarbonate buffer, pH 7.5, and then resuspended in Eagle's medium as before. The procedure yielded cell suspensions of hepatocytes with 90–95% viability as measured by Trypan Blue exclusion.

Incubation procedure

Cell suspensions were incubated with [^{125}I]monoiodoinsulin in the presence of various concentrations of unlabelled hormone at 30°C. The incubation media were constantly gassed. At various times triplicate samples of the incubation media were taken to determine binding and degradation. Separation of cell-bound from free [^{125}I]iodoinsulin was by centrifugation through di-n-butyl phthalate/dinonyl phthalate oil (2:1, v/v) in a Beckman Microfuge. Cell pellet and supernatant radioactivity were counted (LKB Wallac Minigamma). Degradation was measured by precipitability of the label in 15% (v/v) trichloroacetic acid. The specific radioactivity of the tracers was determined by radioimmunoassay (Sönksen, 1976). Controls containing cells but no insulin, with and without bacitracin, were incubated in parallel for 30 min. The cells were spun down (100 *g* for 5 min) and the cell-free supernatants reincubated with labelled insulin for a further 60 min. The amount of degradation in the cell-free supernatant was determined by precipitability in 15% trichloroacetic acid.

Cell-associated radioactivity

Samples (200 μl) of the incubation media were centrifuged (100 *g* for 5 min at 4°C), the supernatants aspirated and the cells resuspended in 200 μl of 6 M-urea/3 M-acetic acid/0.1% Triton X-100. The cells were frozen and thawed in this solution to extract the radioactivity. The degradation of cell-associated radioactivity was measured by precipitability in 15% trichloracetic acid.

Results

Bacitracin had minimal effects on binding of [^{125}I]iodoinsulin over 30 min at 30°C, but markedly decreased the rate of degradation (Figs. 1*a* and 1*b*). Control incubations, as described in the Materials and methods section, showed that the percentage degradation of insulin after 30 min preincubation of cells alone followed by reincubation of the cell-free supernatant with [^{125}I]iodoinsulin was 0.42 ± 0.22% per 10^6 cells after 30 min and 0.91 ± 0.36% per 10^6

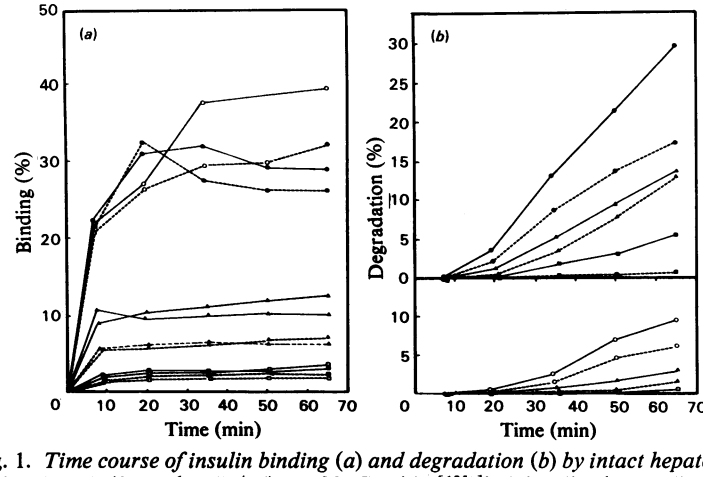

Fig. 1. *Time course of insulin binding (a) and degradation (b) by intact hepatocytes*
Hepatocytes were incubated (3 × 10^6 cells/ml) at 30°C with [^{125}I]iodoinsulin (sp. radioactivity 324 μCi/μg) at 0.14 nM in the absence (●——●) or presence (○——○) of bacitracin (0.88 mM) and with various concentrations of unlabelled hormone (0.78 nM, ●----●; 6.7 nM, ▲——▲; 23 nM, ▲----▲; 0.23 μM, ■——■; 3.1 μM, ■----■;) in the absence or presence (corresponding open symbols) of bacitracin. Bound insulin was determined by centrifugation of the cells through oil and degradation was measured by precipitability in trichloroacetic acid.

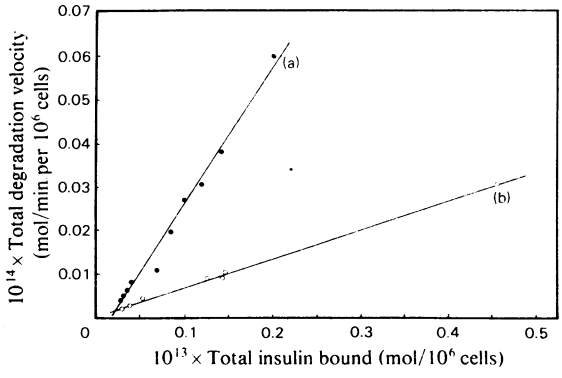

Fig. 2. *Relationship between insulin binding and degradation at low hormone concentrations*

Hepatocytes were incubated for 30min at 30°C with various concentrations (0.02–0.2nM) of [^{125}I]iodoinsulin in the absence (a, ●) and in the presence (b, ○) of bacitracin (0.88mM). The degradation velocity was estimated in each case by regression analysis of the rate of increase in amount degraded with time. The points represent pooled data from three experiments.

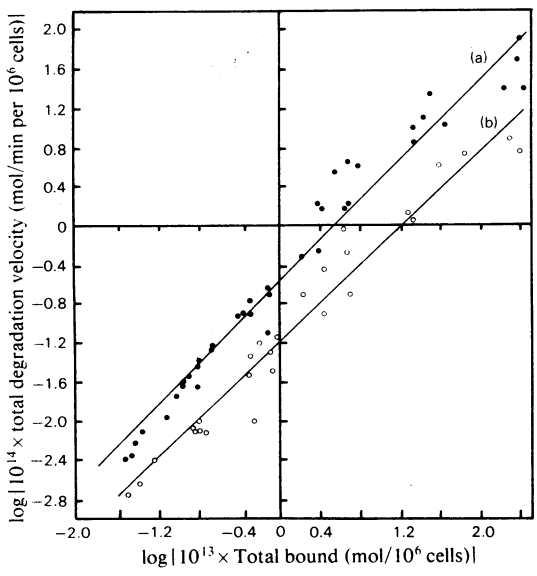

Fig. 3. *Log/log plot of total degradation velocity versus total insulin bound*

Hepatocytes were incubated for 30min at 30°C with insulin in the absence (a, ●) and in the presence (b, ○) of bacitracin (0.88mM). The points represent data from eight experiments in which the insulin concentration was increased by addition of unlabelled hormone (0.1nM–3.0 μM) and by dilution of tracer (0.02–0.2nM) as in Fig. 2.

cells after 60min. Although not directly comparable kinetically, the total percentage degradation of [^{125}I]iodoinsulin incubated with cells was 4.39 ± 0.67 and 9.61 ± 1.25% per 10^6 cells after 30 and 60min respectively. With these low extents of extracellular degradation, the addition of unlabelled hormone progressively decreased both binding and total degradation. The mean values ± S.E.M. ($n = 5$) for the K_d for binding and K_i for degradation were 3.95 ± 1.34nM and 4.74 ± 0.06nM respectively in the absence of bacitracin and 3.15 ± 0.78nM and 5.47 ± 2.40nM in the presence of bacitracin.

Fig. 2 shows a linear relationship between total degradation velocity and total amount of insulin tracer bound at steady-state (30min). The regression equation for line (a) in Fig. 2 is $y = 0.37x - 0.006$ ($r = 0.99$) and for line (b) is $y = 0.067x + 0.0002$ ($r = 0.99$). The rate constant (K_{ap}) relating degradation velocity to a given amount of insulin bound at steady-state is 0.032min^{-1} in the absence of bacitracin and 0.0067min^{-1} in the presence of bacitracin.

Experiments in which a wide range of ligand concentrations was achieved by the addition of various amounts of unlabelled insulin are shown in Fig. 3. The total degradation velocity and total insulin bound are plotted as $\log_{10}$ values in order to cover the range of concentrations used. The regression equation for line (a) is $y = 1.016x - 0.55$ ($r = 0.98$) and for line (b) is $y = 0.959x - 1.14$ ($r = 0.98$). Since the data remain linear over the range studied and the slopes of the regression lines are close to 1.0, the degradation velocity is again shown to be directly proportional to the total bound hormone. From these data K_{ap} is 0.028 min^{-1} without and 0.0079min^{-1} with bacitracin.

When the cell-associated radioactivity was extracted and analysed by precipitability in trichloroacetic acid, it was found (Fig. 4) that bacitracin decreased the fraction of cell-associated [^{125}I]iodoinsulin degraded (soluble in trichloroacetic acid) in a time-dependent manner, and the amount degraded was a smaller percentage of the amount bound.

Discussion

It has been possible to examine the direct effect of bacitracin on cell-mediated insulin degradation, since the preparations of isolated hepatocytes used did not leak large amounts of degrading activity into the medium. Minimal extracellular degrading activity was achieved by constant gassing of the incubation media with O_2/CO_2 to maintain the pH of the bicarbonate-buffered cell suspensions at 7.4 and also by the use of fatty acid-free albumin. The explana-

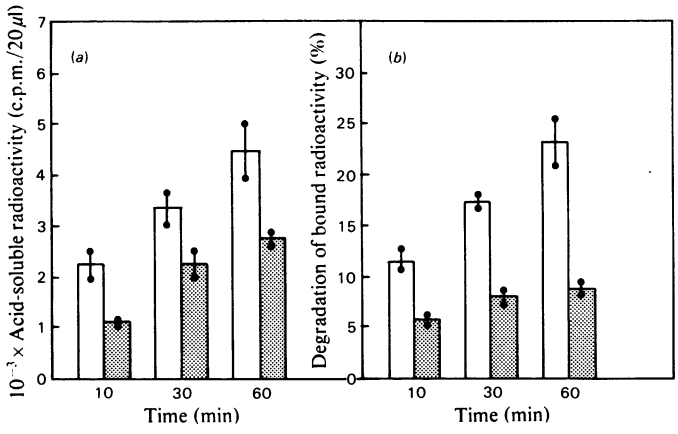

Fig. 4. *Degradation of cell-associated radioactivity*

The extent of degradation was determined after various times of incubation in the absence (□) and presence (■) of bacitracin (0.88 mM). Degradation was determined by trichloroacetate solubility of the radioactivity sedimented with the cell pellet as shown in (*a*). The results are also shown expressed as percentage degradation of bound radioactivity (*b*).

tion for the albumin effect is not clear; there is evidence to suggest that free fatty acids may damage the plasma membrane (Drainas *et al.*, 1981) under certain conditions.

Under conditions of minimal extracellular degrading activity, bacitracin did not alter the time course of binding or the total amount bound over incubation times of 30 min, but markedly decreased the degradation velocity. In agreement with the results of Terris & Steiner (1975), the degradation velocity was found to be directly proportional to the amount bound and $K_{ap} = 0.028\,min^{-1}$ in the absence of bacitracin, which was close to their value of $0.03\,min^{-1}$ calculated over a wide range of insulin concentrations. A lag period of 10 min was also observed before the appearance of measurable degradation products. Furthermore, the K_d for insulin binding and K_i for degradation were similarly of the order of 10^{-9} M, again suggesting that bound insulin is the substrate for degradation.

The values for K_d and K_i were not changed by the presence of bacitracin, but the value for K_{ap} was decreased to $0.0079\,min^{-1}$, indicating that the processing of bound insulin was inhibited. This was reflected by an accumulation of cell-associated radioactivity with longer times of incubation (30–60 min), and a greater proportion of this label was precipitable by trichloroacetic acid.

Commercial bacitracin, produced from *Bacillus licheniformis*, is a group of closely related polypeptide antibiotics designated bacitracin A, B, C etc., of which the predominant form is bacitracin A. The results obtained were no different with two other preparations of bacitracin (results not shown).

Bacitracin inhibits some hydrolytic enzymes such as subtilisin and leucine aminopeptidase (Makinen, 1972). For this reason, it was used to inhibit glucagon inactivation in liver plasma membranes, which had high degrading activity (Desbuquois *et al.*, 1974), and has subsequently been used in many studies of insulin binding and processing in varous systems. It was shown that bacitracin inhibits glutathione–insulin transhydrogenase (Roth, 1981), which had been purified from rat liver, in a manner comparable with the inhibition of insulin degradation found with rat liver lysate. There is, in addition, evidence to show that bacitracin forms complexes with polyprenyl phosphates, thereby inhibiting their incorporation, as precursors, into several biosynthetic pathways, such as sterol (Stone & Strominger, 1972), ubiquinone (Schechter *et al.*, 1974) and glycoprotein (Siewart & Strominger, 1967) biosynthesis. All these effects of bacitracin may alter cell-membrane properties and turnover of membrane constituents.

Studies on receptor-mediated endocytosis of α_2-macroglobulin (Davies *et al.*, 1980) have shown that bacitracin inhibits transglutaminase activity, which is thought to be a controlling factor in receptor clustering before endocytosis and internalization of the ligand. The effect of bacitracin may not, however, be confined to the cell surface.

The present results imply that studies of the processing and internalization of insulin by isolated cells done in the presence of bacitracin at the concentrations required to inhibit extracellular degradation may not reflect physiological conditions, since the cell-mediated processing of insulin may

itself be inhibited. The mode of action of bacitracin in this respect is not known, but all the effects described above are compatible with some membrane interaction. Clarification of the mechanism by which bacitracin exerts these effects may throw light on the nature of cell-mediated insulin processing.

We thank the British Diabetic Association and the St. Thomas's Hospital Research (Endowment) Committee for financial support, and acknowledge the technical assistance of R. Islam.

References

Berry, M. N., Hamilton, P. L., Severinghaus, E. M. & Williams, M. C. (1974) in *Alfred Benzon Symposium VI: Regulation of Hepatic Metabolism* (Lundqvist, F. & Tygstrup, N., eds.), pp. 790–795, Munksgaard, Copenhagen

Caro, J. R. & Armatruda, J. M. (1980) *J. Biol. Chem.* **255**, 10052–10055

Carpentier, J. L., Gorden, P., Freychet, P., LeCam, A. & Orci, L. (1979) *J. Clin. Invest.* **63**, 1249–1261

Davies, J. A., Davies, D. R., Levitzki, A., Maxfield, F. R., Milhaud, P., Willingham, M. C. & Pastan, I. H. (1980) *Nature (London)* **283**, 162–167

Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120

Drainas, D., Harvey, E., Lawrence, A. J. & Thomas, A. (1981) *Eur. J. Biochem.* **114**, 239–245

Duckworth, W., Runyan, K., Wright, R. K., Halban, P. A. & Solomon, S. S. (1981) *Endocrinology* **108**, 1142–1147

Freychet, P., Kahn, C. R., Roth, J. & Neville, D. M., Jr. (1972) *J. Biol. Chem.* **247**, 3953–3960

Gammeltoft, S. & Gliemann, J. (1973) *Biochim. Biophys. Acta* **320**, 16–32

Gammeltoft, S., Kristenson, L. O. & Sestoft, L. (1978) *J. Biol. Chem.* **253**, 8406–8413

Gliemann, J. & Sonne, O. (1978) *J. Biol. Chem.* **253**, 7857–7863

Greenwood, F. C., Hunter, W. H. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123

Hammond, J. M. & Jarrett, L. (1975) *Diabetes* **24**, 1011–1019

Kahn, C. R., Freychet, P., Roth, J. & Neville, D. M., Jr. (1974) *J. Biol. Chem.* **249**, 2249–2257

Linde, S., Hansen, B. & Lernmark, A. (1980) *Anal. Biochem.* **107**, 165–176

Makinen, K. K. (1972) *Int. J. Protein Res.* **4**, 21–28

Olefsky, J. M., Johnson, J., Liu, F., Edwards, P. & Baur, S. (1975) *Diabetes* **24**, 801–810

Roth, R. A. (1981) *Biochem. Biophys. Res. Commun.* **98**, 431–439

Schechter, N., Momose, K. & Rudney, H. (1974) *Biochem. Biophys. Res. Commun.* **48**, 833–839

Seglen, P. (1973) *Exp. Cell Res.* **82**, 391–398

Siewart, G. & Strominger, J. L. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 767–773

Sönksen, P. H. (1976) in *Hormones in Human Blood: Detection and Assay* (Antoniades, H. N., ed.), Harvard University Press

Stone, J. K. & Strominger, J. L. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1287–1289

Terris, S. & Steiner, D. F. (1975) *J. Biol. Chem.* **250**, 8389–8398