Effect of Bacitracin on Binding and Processing of Insulin by Established Renal Cell Line

CHANA YAGIL, BRUCE H. FRANK, AND RALPH RABKIN

The effect of bacitracin on the binding and processing of ^{125}I-labeled insulin was studied in a proximal tubular epithelium–like opossum kidney cell line. This cultured cell line handles insulin in a manner comparable to the in vivo situation, which requires membrane binding, internalization, and intracellular degradation. The addition of bacitracin inhibited insulin degradation significantly and delayed the time of appearance of products in the medium (22 min) compared with control cells (14 min). Maximum total cell-associated radioactivity increased from 1.5 ± 0.19% in the control cells to 2.5 ± 0.17% in the treated cells. Separation of cell membrane from internalized radioactivity was achieved by acid washing and showed no change in membrane-bound radioactivity or rate of internalization, but a significant increase in intracellular radioactivity was noted. Gel-filtration chromatography revealed that this was due to an accumulation of chromatographically intact insulin. Accordingly, we conclude that bacitracin inhibits insulin degradation in cultured kidney cells by perturbing the intracellular processing of insulin, not by altering the binding or internalization of the hormone or by inhibiting the release of small degradation products. Because of the multiple actions of this agent, the exact site in these kidney cells at which intracellular degradation is inhibited remains to be established. However, in contrast to studies with lysosomes isolated from cells of other tissues, this study showed that when lysosomes isolated from rat renal cortex were exposed to bacitracin, insulin degradation was inhibited markedly (81%). *Diabetes* 37:800–805, 1988

It is widely recognized that the kidney plays a major role in the metabolism of insulin (1). Insulin removed from the circulation by glomerular filtration binds to the apical surface of the proximal tubular epithelium, is then subjected to endocytosis, and is finally degraded within the cell. Internalization appears to be partially mediated by the insulin receptor (2). Pursuing our studies of the renal metabolism of insulin, we recently identified a cultured kidney cell line that provides a good model for studying the renal uptake and processing of insulin (3). This cell line, derived from the opossum kidney (OK), has proximal tubular epithelium–like characteristics (4–6) and takes up and processes insulin in a manner analogous to the in vivo situation. In this respect the OK cells possess insulin-specific receptors on the cell surface, and binding to these receptors is followed by endocytosis and degradation within the cell. Release of insulin-degrading enzymes into the extracellular medium is negligible (3).

To further explore the uptake and processing of insulin by these cultured kidney cells, we used an established inhibitor of insulin metabolism, i.e., bacitracin. This cyclic polypeptide has been widely used in the study of insulin metabolism, originally on the basis that its action was limited to inhibition of extracellular degradation (7,8). However, studies with several different cell types, including adipocytes, hepatocytes, and fibroblasts, have indicated that the action of bacitracin is not limited to an extracellular site. Indeed, these studies have varied in their results, and consequently, bacitracin action has been attributed many sites. Bacitracin's inhibition of degradation has been said to occur in the cell, plasma membrane, or extracellular fluid (7–11). Additionally, there are reports that bacitracin inhibits both the internalization and the dissociation of membrane-bound hormone (10,12). The reasons for these conflicting reports are not always apparent, but in some instances they reflect differences in the manner in which cell types process insulin (7). Accordingly, in this study we examined the effect of bacitracin on insulin binding and processing in the cultured OK cell with the pur-

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pose of elucidating the general site of bacitracin action in these cells to further our understanding of renal epithelial cell insulin metabolism.

MATERIALS AND METHODS

Human A14-[^{125}I]monoiodoinsulin was prepared according to Frank et al. (13). When it was used, <2% of the radioactivity was soluble in trichloroacetic acid (TCA). Bovine serum albumin (BSA) fraction V, essentially fatty acid free, was obtained from Miles Laboratories (Elkhart, IN), and bacitracin was obtained from Sigma (St. Louis, MO). Reagent-grade chemicals were used.

OK cells from passages 65–85 were plated on 35-mm plastic dishes (~0.5×10^6 cells/dish) in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% newborn calf serum, L-glutamine (2 mM), penicillin (120 U/ml), streptomycin (72 μg/ml), and hydrocortisone (50 nM). Cultures were maintained at 37°C in the presence of 5% CO_2 and 95% air and reached confluency within 3–4 days.

Before study, the culture medium was removed and replaced with incubation medium, which consisted of a Krebs-Ringer solution containing 5 mM glucose, 1% BSA, and 16 mM HEPES (KRH) or 16 mM Tris (KRT) at pH 7.4. ^{125}I-labeled insulin, 2×10^{-11} M, was added, and the monolayers were incubated at 37°C in the presence of 0.6 mM bacitracin. Monolayers incubated in parallel in the absence of bacitracin, described in a companion study (3), served as controls. Nonspecific binding of ^{125}I-insulin measured in the presence of 10^{-5} M unlabeled pork insulin was <5% of the total cell-associated ^{125}I-insulin.

The distribution of cell-associated ^{125}I-insulin between the cell surface and the interior of the cell was measured by the acid-wash technique, which is a well-established procedure (14–16) that has been reported in detail from this laboratory in a companion study (3). In brief, after incubation with ^{125}I-insulin, monolayers are washed five times with a cold saline solution to remove free ^{125}I-insulin. The cells are then incubated for 10 min at 4°C, with the incubation medium adjusted to pH 2.8. The medium is then removed, and the eluted acid-sensitive radioactivity is counted. Acid-resistant radioactivity is determined after solubilization of the cells in 0.1% sodium dodecyl sulfate. This acid-wash procedure removes 94% of the surface-bound radioactivity. The acid-sensitive radioactivity is taken to be cell membrane–bound material, and acid-resistant radioactivity is taken to be internalized material.

Degradation of ^{125}I-insulin was determined from the formation of radioactivity soluble in TCA. This was achieved by adding an equal volume of 10% TCA to the sample and then separating supernatant from precipitate by centrifugation. The percentage of degraded insulin was determined from the increase in TCA-soluble radioactivity over that observed in control dishes containing medium but no cells. We have found that although this TCA method underestimates insulin degradation, it correlates well with an antibody method used in this laboratory (17). Radioactivity was also analyzed by chromatography on a Sephadex G-50 fine column (0.9×60 cm) with 1 M acetic acid containing 0.1% BSA as the eluent. At least 95% of the radioactivity added was recovered from the columns.

To evaluate the effect of bacitracin on lysosomal insulin-degrading activity, lysosomes were isolated from rat kidney cortex and incubated with ^{125}I-insulin in the presence or absence of bacitracin ($n = 3$). The isolation procedure, which involves separation on a linear sucrose gradient, was similar to that described by Hjelle et al. (18) except for the following modifications. First, homogenized rat kidney cortex was used as starting material. Second, the postnuclear supernatant was subjected to centrifugation at $6000 \times g$ for 10 min, and the fluffy upper pellet was removed and layered onto the sucrose gradient that extended from 1.10 to 1.26 g/ml. After centrifugation, 2-ml fractions were collected and processed for the lysosomal marker enzyme *N*-acetyl-β-glucosaminidase (19). Protein and marker enzymes for other organelles were measured as previously described (2,20). The lysosomal fractions compared with homogenate were enriched 13-fold in *N*-acetyl-β-glucosaminidase. Enrichment in other enzymes were as follows: lactate dehydrogenase (cytosol), 0; cytochrome oxidase (mitochondria), 0.63; and alkaline phosphatase (apical tubular plasma membrane), 0.2. Fractions enriched in lysosomes were pooled, and the pH was adjusted to 3.5 with a sodium citrate–citric acid buffer, final concentration 50 mM. After the addition of

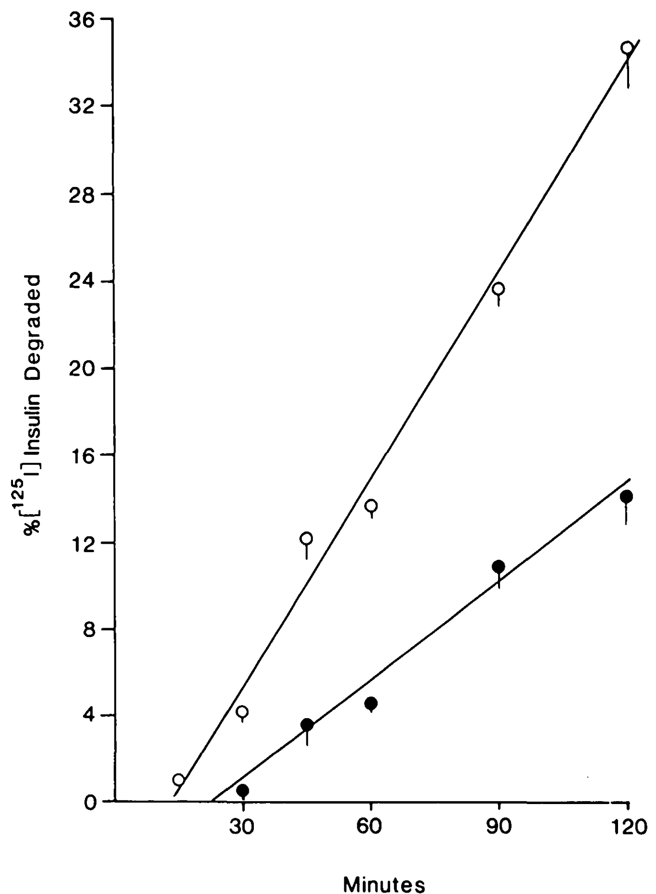

FIG. 1. Effect of bacitracin on ^{125}I-labeled insulin degradation. Monolayers of cultured kidney cells were incubated at 37°C with ^{125}I-insulin either alone or with 0.6 mM bacitracin. Samples of medium were assayed for release of trichloroacetic acid–soluble radioactivity. Degradation products were not detected in 15-min samples from bacitracin-treated group. Values are means ± SE from 4 monolayers pooled from 2 experiments. For control (○) versus bacitracin-treated (●) cells, $P < .005$.

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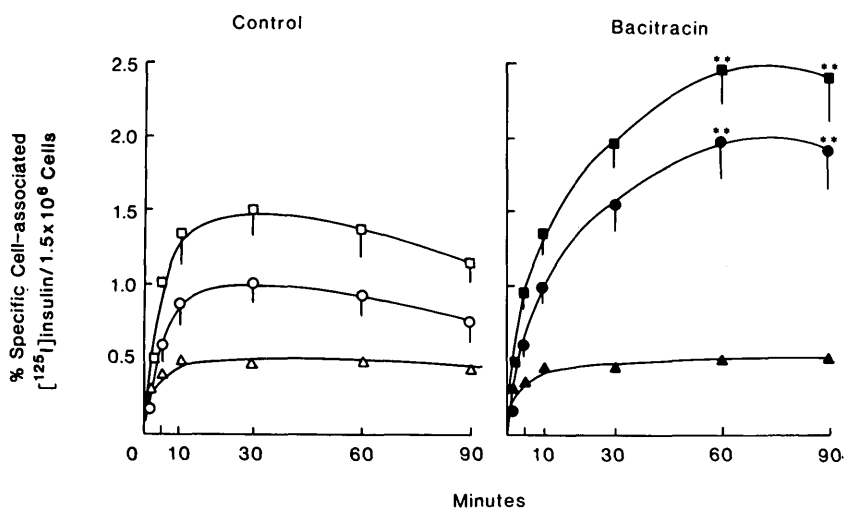

FIG. 2. Effect of bacitracin on association of ^{125}I-labeled insulin with cultured kidney cells. Monolayers were incubated with ^{125}I-insulin (4×10^{-11} M) at 37°C in presence or absence of bacitracin. In some experiments 0.6 mM bacitracin was added. Separation of membrane-bound from internalized radioactivity was achieved by acid washing. Values are means ± SE from 6 monolayers pooled from 3 experiments and are corrected for nonspecific association ($P < .005$ vs. control cells). *Squares*, total; *circles*, internalized; *triangles*, membrane-bound radioactivity.**

deoxycholate (10 mg/dl) and BSA (300 mg/dl), aliquots were incubated at 4°C for 15 min. ^{125}I-insulin (1.7×10^{-10} M) was then added, and the lysosomes (44–72 μg protein/500 μl) were incubated at 37°C in the absence or presence of bacitracin (0.6 mM). Parallel tubes containing ^{125}I-insulin, but no lysosomes, served to correct for any spontaneous degradation. Degradation was measured by the release of TCA-soluble radioactivity.

DATA ANALYSIS

All results were corrected for 1.5×10^6 cells and are expressed as means ± SE. Because data were obtained from monolayers incubated in pairs, analysis was carried out with Student's paired *t* test. To allow for multiple comparisons with the controls, Bonferroni's adjustment was made, and $P < .01$ was regarded as significant (21). To achieve homogeneity of variances, data were transformed to logarithmic values before statistical analysis.

RESULTS

The time course of ^{125}I-insulin degradation by monolayers of OK cells incubated in the presence or absence of bacitracin is shown in Fig. 1. Bacitracin produced a significant depression of cell-associated degradation ($P < .005$) and slowed the appearance of products in the medium. Regression analysis of the appearance of TCA-soluble radioactivity yielded an intercept of 14 ± 1.7 min in the control cells, but in the experimental cells it was 22 ± 0.7 min ($P < .005$). These values were assumed to reflect the time taken to bind, internalize, degrade, and release the products of degradation. In a companion study we observed the negligible release of insulin-degrading activity into the incubation medium. Cell-free medium previously exposed to cells for 2 h degraded 1.2% of added insulin/h (3). To explore the site of bacitracin action, cell-associated, internalized, and membrane-bound radioactivity was determined over time in monolayers incubated with or without bacitracin (Fig. 2). When bacitracin was included in the incubation medium, specific cell-associated radioactivity increased significantly compared with that of controls. At maximum, cell-associated radioactivity in control cells averaged 1.52 ± 0.19% (30 min) and in the experimental group, 2.48 ± 0.19% (60 min). Examination of the data in Fig. 2 reveals that this is a consequence of an increase in the amount of radioactivity in the intracellular

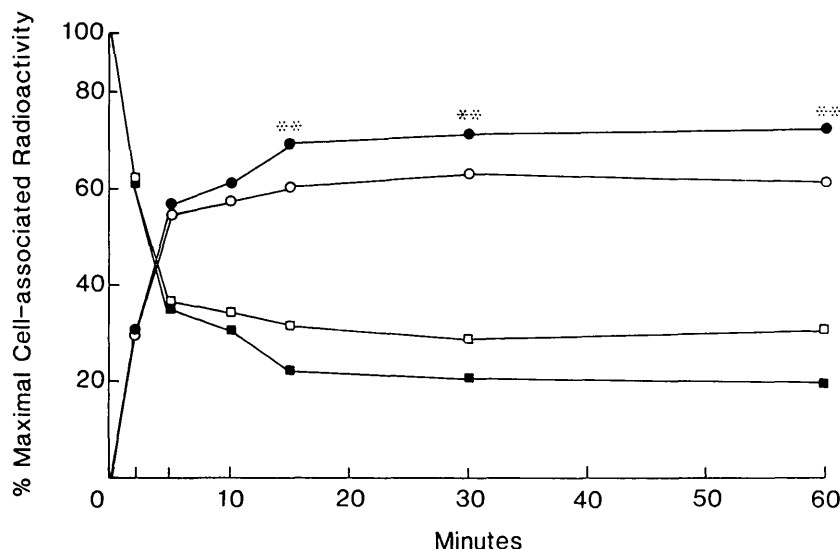

FIG. 3. Effect of bacitracin on fractional distribution of cell-associated radioactivity. Data are derived from experiments for Fig. 2 ($P < .005$ vs. control). ●, Internalized plus bacitracin; ○, internalized control; □, membrane-bound control; ■, membrane-bound plus bacitracin radioactivity.**

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compartment. There was no significant difference in the membrane-bound radioactivity in the two groups.

Analysis of the fractional distribution of radioactivity between the membrane-bound and the intracellular compartments (Fig. 3) shows that in the bacitracin-treated and control cells the rates of internalization were essentially identical during the first 5 min. Under both circumstances, fractional distribution of radioactivity reached a steady state by 15 min. This result differs from our reported findings on the effect of lowering the temperature to 25°C (3); at this temperature, degradation is also reduced, but the rate of internalization is slowed and a steady state is reached after 30 min. Figure 3 illustrates that bacitracin altered the steady-state fractional distribution of cell-associated radioactivity significantly. In the control cells the intracellular fraction reached a maximum of 69 ± 2%, whereas in the presence of bacitracin, it was 79 ± 1% ($P < .005$). From these experiments it appears that although the inhibition of degradation by bacitracin is associated with an increase in intracellular radioactivity, this agent does not alter the absolute amount of radioactivity bound to the cell surface (Fig. 2) or the rate of internalization (Fig. 3).

The radioactivity remaining in the cells after acid washing was extracted by washing three times with 3 M acetic acid containing 0.1% Triton X-100. This procedure extracted ~98% of the radioactivity, which was then chromatographed on a Sephadex G-50 column. Figure 4 depicts the elution profile of the acid-resistant radioactivity extracted from control and bacitracin-treated cells. Radioactivity eluted predominantly in two peaks, with an additional small amount of radioactivity coeluting with blue dextran (void volume). Of the two major peaks, the large peak eluted in the same position as intact insulin, and the small peak eluted in the same position as monoiodotyrosine. Compared with the control, bacitracin was associated with an increase in the peak coeluting with intact insulin and a decrease in the peak coeluting with monoiodotyrosine. Insulin-peak material comprised 55% of the intracellular radioactivity in each of two control monolayers and 70 and 81% of the radioactivity in two bacitracin-treated monolayers. From the analysis it is evident that the bacitracin increase in acid-resistant radioactivity shown in Fig. 2 results from an increase in chromatographically intact ^{125}I-insulin and not from an accumulation of small degradation products. In view of the data presented earlier, we conclude that bacitracin inhibits insulin degradation by perturbing the intracellular processing of insulin, not by altering the binding or internalization of the hormone or by inhibiting the release of degradation products.

In the experiments designed to determine whether bacitracin affects lysosomal insulin-degrading activity, a marked inhibitory action was observed. Degradation of insulin by isolated control lysosomes averaged 14.0 ± 0.02% · mg^{-1} protein · min^{-1}; in the presence of 0.6 mM bacitracin, degradation fell to 1.4 ± 0.2% ($P > .001$). This difference represents 81 ± 3% inhibition of degradation.

DISCUSSION

This study, examining the effect of bacitracin on the uptake and processing of insulin by a cultured renal epithelial cell line, provides new insights into the action of this agent and the intracellular metabolism of insulin in cultured kidney cells. This cyclic polypeptide has been used widely in the study of receptor-mediated endocytosis and cellular processing of insulin. Bacitracin was originally used to inhibit degrading activity released by cells, and it was assumed that it did not directly affect cellular insulin degradation. More recent studies have shown that bacitracin also acts directly on the cell, but there is a lack of agreement on the exact site of action (7–12). This lack of consensus can largely be attributed to differences in the manner in which various cell types interact with and process insulin. In some instances this disagreement may also reflect differences in results obtained from cell-free systems and from intact cells. Despite the drawbacks of in vitro data, it is worth noting that bacitracin has a pronounced inhibitory action on the major insulin-degrading enzymes, insulin protease and glutathione-insulin transhydrogenase (22–24), and, as demonstrated in this study, lysosomal insulin-degrading activity. Our observation that bacitracin (0.6 mM) inhibited renal lysosomal insulin-degrading activity by 81% differs from the small or negligible

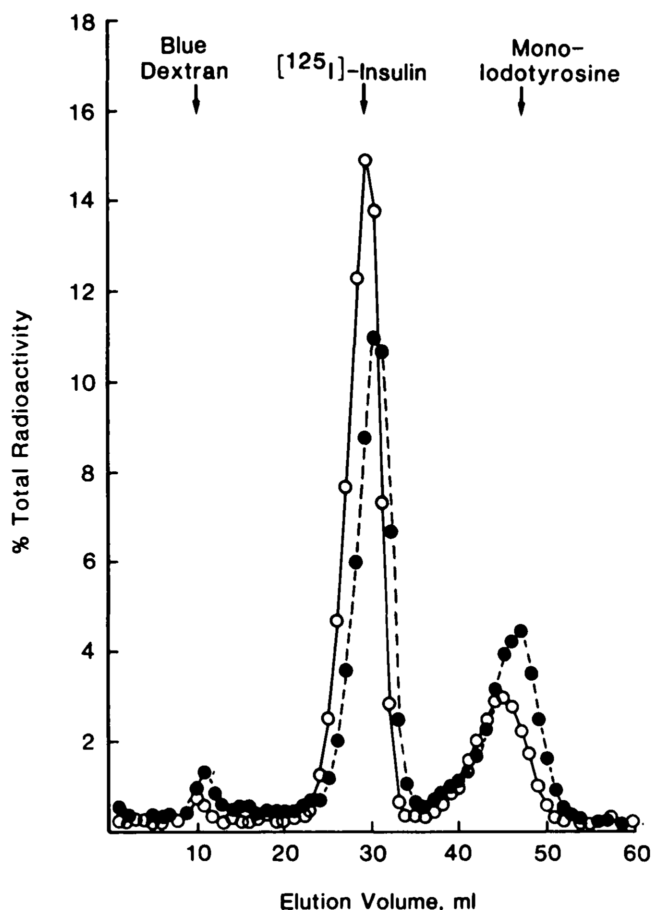

FIG. 4. Gel-filtration profile of acid-resistant (internalized) cell-associated radioactivity. Monolayers were incubated in absence (controls) or presence of 0.6 mM bacitracin with ^{125}I-labeled insulin for 1 h at 37°C. Monoiodotyrosine (2 mM) was included in incubation medium to prevent deiodination of small radiolabeled products. Cell-associated radioactivity resistant to acid wash from control (●) or bacitracin-treated (○) cells was subjected to analysis on Sephadex G-50.

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effect noted by others with lysosomes prepared from different tissues. At similar bacitracin concentrations, the insulin-degrading activity of lysosomes prepared from adipocytes was inhibited 19% (25), but fibroblast lysosomal activity did not change significantly (11).

Although some studies with intact hepatocytes suggest that bacitracin inhibits insulin degradation and internalization at the level of the cell membrane (9,10), Peavy et al. (8) concluded that bacitracin inhibits degradation within the cell. This conclusion was based on the finding that bacitracin increased cell-associated insulin intermediate products. In studies of fibroblasts Stentz et al. (11) and Kitabchi and Stentz (26) also noted an increase in cell-associated insulin intermediates when bacitracin was present. But they concluded that this result was due to inhibition at a cell membrane site. However, in both studies no distinction was made between surface-bound and intracellular products. In an attempt to resolve these disparate reports, Gansler et al. (7) studied the effect of bacitracin on the association and degradation of insulin by six cell types. These included adipocytes, hepatoma cells, and fibroblasts. In all cell types, bacitracin inhibited insulin degradation when studied at temperatures between 24 and 37°C. However, cell-associated insulin was not uniformly affected. It was increased in rat adipocytes but decreased in the other cell types. In the latter, the decrease in cell-associated insulin was due to a decrease in intracellular insulin. In contrast, in rat adipocytes the increase in cell-associated radioactivity was due to a proportional increase in both intracellular and membrane-bound insulin. These researchers concluded that there was cell-type–specific variability in the response to bacitracin. This may represent differences in the manner in which cells process insulin.

In this study we have demonstrated that in a proximal renal tubular epithelium–like cell line, bacitracin inhibits insulin degradation at an intracellular site. This conclusion is supported by the finding that bacitracin did not affect insulin binding to the cell membrane and did not affect internalization but did cause an increase in intracellular radioactivity. Furthermore, in bacitracin-treated cells compared with control cells, a greater proportion of this intracellular radioactivity eluted in the same position as intact insulin on gel-filtration chromatography (Fig. 4). Although this radioactive material may include slightly modified insulin (22,28), it does indicate that the accumulation of intracellular radioactivity was due to a failure of processing rather than a failure of the export of small degradation products.

The precise site at which bacitracin acts is not apparent from these studies. Kidney epithelial cells probably readily internalize this cyclic polypeptide via endocytosis and deliver it to lysosomes (29). If this process occurs, and if bacitracin inhibits lysosomal hydrolytic function in these cultured kidney cells as it does with lysosomes from rat renal cortex, then the site of bacitracin action is readily accounted for. However, bacitracin also profoundly inhibits insulin protease and glutathione-insulin transhydrogenase activity and could thereby inhibit extralysosomal cellular insulin degradation (22–24). A further consideration is the possibility that bacitracin may, by a more general disruptive effect on cellular function, impair intracellular trafficking of insulin after it is internalized. Indeed, bacitracin is a known nephrotoxin that can alter cell metabolism (30).

Studies identifying the exact site at which bacitracin exerts its action should help clarify our understanding of the intracellular processing of insulin in cultured kidney cells. Moreover, in view of the cell-type–specific variability in reponse to bacitracin, this approach may uncover differences in the processing of insulin by cells derived from different tissues.

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