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Epidermal growth factor stimulation of DNA synthesis is potentiated by compounds that inhibit its clustering in coated pits

(receptors/endocytosis/amines/bacitracin/transglutaminase)

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ABSTRACT We have used inhibitors of receptor-mediated endocytosis to investigate the mechanism and function of epidermal growth factor uptake by cultured cells. When rhodamine-labeled epidermal growth factor is bound to cell surface receptors on confluent monolayers of BALB/c 3T3 cells, it rapidly collects in cell surface clusters and is internalized. The clustering of occupied receptors requires Ca^{2+} and is inhibited by primary alkylamines; both of these properties are shared by the enzyme transglutaminase (R-glutaminyl-peptide:amine γ-glutamyl-yltransferase, EC 2.3.2.13). In Chinese hamster ovary cell extracts, methylamine inhibits 25–50% of the transglutaminase activity with a K_i of 0.2 mM, and it inhibits the remaining transglutaminase activity with a K_i of 20 mM. Clustering is almost completely inhibited by 10 mM methylamine. The polypeptide antibiotic bacitracin inhibits clustering of rhodamine-labeled epidermal growth factor or α_2-macroglobulin at 0.7 mM, and it inhibits approximately 40% of the transglutaminase activity in Chinese hamster ovary cells with a K_i of 0.03 mM. Fluorescent ligands bound to cell surface receptors in the presence of bacitracin form clusters within 30 min after bacitracin is removed from the culture medium. These results indicate that a transglutaminase-like enzyme may be required for the clustering and subsequent internalization of occupied receptors. The effects of 10 mM methylamine and 0.7 mM bacitracin on epidermal growth factor stimulation of DNA synthesis were examined. The stimulation of DNA synthesis by epidermal growth factor was increased 2- to 7-fold in the presence of methylamine or bacitracin. Alone, methylamine or bacitracin increased DNA synthesis 1.1- to 3-fold. The stimulation of DNA synthesis resulting from the simultaneous presence of the hormone and the clustering inhibitor was always greater than the sum of the stimulations produced by the hormone and the clustering inhibitors alone. The potentiation of epidermal growth factor activity by clustering inhibitors suggests that the hormone acts at the cell surface. We propose that rapid internalization of occupied receptors via coated pits may be a mechanism to limit the response to hormones.

It is widely accepted that the initial step in polypeptide hormone action is the binding of the hormone to a cell surface receptor (reviewed in ref. 1). After binding, these hormones are internalized via receptor-mediated endocytosis (2, 3). It has been difficult to determine what role, if any, internalization plays in hormone action. One widely held idea is that the delivery of hormones or receptors to intracellular sites may be required for some actions of hormones. Effects that have a long lag time (4), such as the stimulation of mitogenesis, are particularly attractive candidates for a mechanism that requires internalization. Other roles for internalization are the down-regulation of hormone receptors in response to ligand binding and hormone destruction (reviewed in ref. 1).

Our approach to this problem has focused on studying the mechanism of receptor-mediated endocytosis in cultured fibroblasts. Based on studies with fluorescent derivatives of insulin, epidermal growth factor (EGF), and α_2-macroglobulin (α_2M) (3, 5–7) and electron microscopic localization of α_2M bound to cell surface receptors (7, 8), we have proposed (8) a model for receptor-mediated endocytosis that may be summarized as follows: (*i*) ligands bind to diffusely distributed, mobile receptors; (*ii*) after ligand binding, the occupied receptors cluster over coated pits in the plasma membrane; (*iii*) the coated pits pinch off from the membrane to form endocytic vesicles that contain the ligands and, probably, their receptors.

We have shown that insulin, EGF, and α_2M collect in the same cell surface clusters and enter cells in the same coated endocytic vesicles (6, 8). Gorden *et al.* (9) have used electron microscopic autoradiography to demonstrate that EGF becomes concentrated on the fibroblast cell surface over coated pits, and Haigler *et al.* (10) have shown that coated pits participate in the uptake of ferritin-conjugated EGF by A-431 epithelioid carcinoma cells. Anderson *et al.* (11) have shown that low density lipoproteins enter cells via coated vesicles.

Although endocytosis via coated pits and coated vesicles appears to be the major pathway for internalization of receptor-bound proteins and polypeptides, there are other mechanisms for endocytosis. In fluid-phase pinocytosis, uncoated regions of the membrane pinch off to form vesicles that contain culture medium (12). This process differs from receptor-mediated endocytosis in that no concentration of proteins occurs before endocytosis. Some occupied receptors may be nonselectively included within these pinosomes (10), but fluid-phase pinocytosis can only account for a small percentage of the uptake of receptor-bound ligands. The half-time for internalization of occupied receptors is less than 10 min (13), whereas the half-time for membrane turnover via fluid-phase pinocytosis is 1 hr or longer (12).

Recently, we found that the clustering and subsequent internalization of occupied receptors required calcium and could be inhibited by primary alkylamines such as methylamine (7). We noted that these properties were shared by the enzyme transglutaminase (R-glutaminyl-peptide:amine γ-glutamyl-yltransferase, EC 2.3.2.13), which catalyzes the formation of ε-(γ-glutamyl)lysine crosslinks between proteins (14). Furthermore, we indicated that inhibition of clustering and internalization might be useful in determining whether receptor-mediated endocytosis is required for hormone action (7).

In this paper we report that, in addition to primary amines, the antibiotic bacitracin is an inhibitor of the clustering of receptors and an inhibitor of transglutaminase. We also show that both bacitracin and methylamine, at concentrations that inhibit clustering, potentiate the stimulation of DNA synthesis by EGF. These results are in accord with the hypothesis that EGF exerts its actions while located on the cell surface.

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Abbreviations: α_2M, α_2-macroglobulin; EGF, epidermal growth factor; CHO, Chinese hamster ovary.

MATERIALS AND METHODS

EGF Stimulation of DNA Synthesis. The incorporation of [^{3}H]thymidine into DNA was used as an assay for the mitogenic activity of EGF (15). BALB/c 3T3 cells were routinely cultured at 37°C in Dulbecco–Vogt's modified Eagle's medium supplemented with 10% (vol/vol) calf serum. The cells were plated in 35-mm Falcon tissue culture dishes at a density of 5×10^5 cells per dish in 3 ml of medium supplemented with 10% calf serum that had been heated to 60°C for 1 hr. The medium was changed every other day for 8–10 days. Two days after the final medium change, 1.5 ml of the conditioned medium was drawn off and saved. At this time, methylamine hydrochloride (10 mM) or bacitracin (0.7 mM) was added to the culture medium of some of the dishes. Thirty minutes later, mouse EGF (1.5 nM) was added to some dishes. At 2.5 hr after the addition of EGF, all dishes, including controls, were rinsed twice with warm Dulbecco's phosphate-buffered saline and the medium was replaced with the conditioned medium that had previously been drawn off. Twenty hours after the EGF had been added, fresh medium containing [^{3}H]thymidine [1 μCi/ml (1 Ci = 3.7×10^{10} becquerels); 0.15 μM] was added to the cells for 2 hr. The cells were then rinsed twice with warm phosphate-buffered saline and incubated with 5% trichloroacetic acid at 4°C for 20 min. After two additional rinses with cold trichloroacetic acid, the cells were dissolved in 0.5 ml of 1 M NaOH and transferred to counting vials. The dishes were rinsed with 0.5 ml of water which was added to the counting vials along with 0.5 ml of 1 M acetic acid and 13.5 ml of scintillation fluid (Aquasol, New England Nuclear). Results shown are the mean of triplicate determinations.

Transglutaminase Activity in Cell Extracts. Transglutaminase activity in Chinese hamster ovary (CHO) cell extracts was measured by using the Ca^{2+}-dependent covalent incorporation of [^{3}H]putrescine into casein in a filter paper assay (16, 17). The assays were run at 37°C in 200 μl of 20 mM Tris-HCl (pH 7.4) containing 2 mg of casein per ml, 5 mM 2-mercaptoethanol, 5 mM $CaCl_2$ or 5 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid, CHO cell extract (protein concentration, 0.5–1.5 mg/ml), 5 mM [^{3}H]putrescine (40 dpm/pmol), and various amounts of inhibitor. The reaction was initiated by the addition of the putrescine, and duplicate aliquots (25 μl) were withdrawn at 3 and 6 min. The aliquots were absorbed on Whatmann 3MM filter paper and immediately dropped in cold 10% trichloroacetic acid. After washing and drying, the filters were placed in liquid scintillator (Econofluor, New England Nuclear) and assayed for radioactivity. Analyses with BALB/c 3T3 cells were run in the same way except that 2.6 μM putrescine (10^5 dpm/pmol) was used and the assay was run for 15 and 30 min. Values of K_i for methylamine and bacitracin were obtained by varying the inhibitor concentration in the presence of 5 mM putrescine. The K_i for putrescine as a substrate for CHO transglutaminase is 5 mM (P. Milhaud, personal communication).

CHO cell extracts were prepared by sonication of cells suspended in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} and supplemented with 0.5 mM EDTA, 1 mM dithiothreitol, and 0.5% Trasylol (Sigma). Extracts were stored frozen at −70°C. BALB/c 3T3 cells were obtained by rinsing confluent monolayers twice with 20 mM Tris-buffered (pH 7.4) saline, followed by one rinse with 0.5 mM EDTA/20 mM Tris, pH 7.4/15 mM mercaptoethanol. The buffer was drawn off, and the cells were scraped from the dish with a rubber policeman and stored on ice for 1 hr. The cells were homogenized by 20 strokes of a Dounce homogenizer.

Binding and Internalization of Rhodamine-EGF. Confluent, quiescent cultures of BALB/c 3T3 cells were obtained as described above. Two days after the last medium change, rhodamine-labeled EGF (10 nM) was added to the medium for 20 min. After the incubation with rhodamine-EGF, the cells were rinsed with warm serum-free medium and fixed with 2% formaldehyde in Dulbecco's phosphate-buffered saline. Where indicated, methylamine or bacitracin was added 20 min before the addition of rhodamine-EGF and was present during the incubation with the hormone.

Materials. Rhodamine-labeled mouse EGF was prepared by Meloy Laboratories according to the method of Schecter *et al.* (18). It consists of rhodamine-labeled lactalbumin (≈8 mol of rhodamine per mol of lactalbumin) coupled to the NH_2-terminal amino acid of EGF. Mouse EGF was from Collaborative Research. [^{3}H]Thymidine and [^{3}H]putrescine were from New England Nuclear. Bacitracin was from Sigma.

RESULTS

Inhibition of Rhodamine-EGF Clustering. Fig. 1*A* shows the punctate pattern of fluorescence that resulted from the clustering and internalization of rhodamine-labeled EGF on BALB/c 3T3 mouse fibroblasts. As shown in Fig. 1*C*, 10 mM methylamine blocked the clustering of rhodamine-labeled EGF under conditions similar to those used to investigate EGF stimulation of DNA synthesis (see below). We have previously shown that methylamine blocks the formation of clusters over coated pits (7).

The polypeptide antibiotic bacitracin has been shown to inhibit the inactivation of glucagon that follows its binding to receptors in isolated membranes (20). It has also been used in studies with enkephalin (21) and insulin (22) to prevent breakdown of those polypeptides after receptor binding. Because these effects might be related to receptor clustering, we examined the effects of bacitracin on the clustering of rhodamine-labeled EGF on the cell surface. As shown in Fig. 1*E*, 0.7 mM bacitracin greatly inhibited the clustering of rhodamine-labeled EGF. We found that 0.07 mM bacitracin only slightly inhibited the clustering of EGF. In contrast to our observations with primary alkylamines (7), preincubation with bacitracin was not required for the inhibition of clustering. This indicates that bacitracin either acts extracellularly or enters the cells more rapidly than do the alkylamines.

Under the conditions used for the DNA synthesis experiments, BALB/c 3T3 cells have a large amount of cytoplasmic autofluorescence. This autofluorescence is roughly proportional to the thickness of the cytoplasm. The nucleus appears dark, and the region surrounding the nucleus is bright. The fluorescence of the rhodamine-labeled EGF is superimposed on this autofluorescence. The bright fluorescent clusters in Fig. 1*A* stand out against the autofluorescent background, but the diffuse fluorescence from rhodamine-labeled EGF in Fig. 1 *C* and *E* is not easily seen against this background. The binding of rhodamine-labeled EGF is not inhibited by bacitracin or methylamine. Rhodamine-labeled EGF bound to cell surface receptors in the presence of methylamine or bacitracin formed clusters within 30 min after the methylamine or bacitracin was removed from the culture medium. No changes in the appearance of cells were observed by phase-contrast microscopy after 3 hr in 0.7 mM bacitracin.

Transglutaminase Activity in Cell Extracts. If, as proposed, a transglutaminase-like enzyme is involved in receptor clustering, then methylamine and bacitracin should inhibit the enzyme in extracts of cultured cells. Using the incorporation of putrescine into casein as an assay, we found that BALB/c cell extracts would incorporate 0.6 pmol/min per mg of protein. Bacitracin (0.7 mM) inhibited approximately 20% of this activity; 10 mM methylamine inhibited 33% of the activity, and

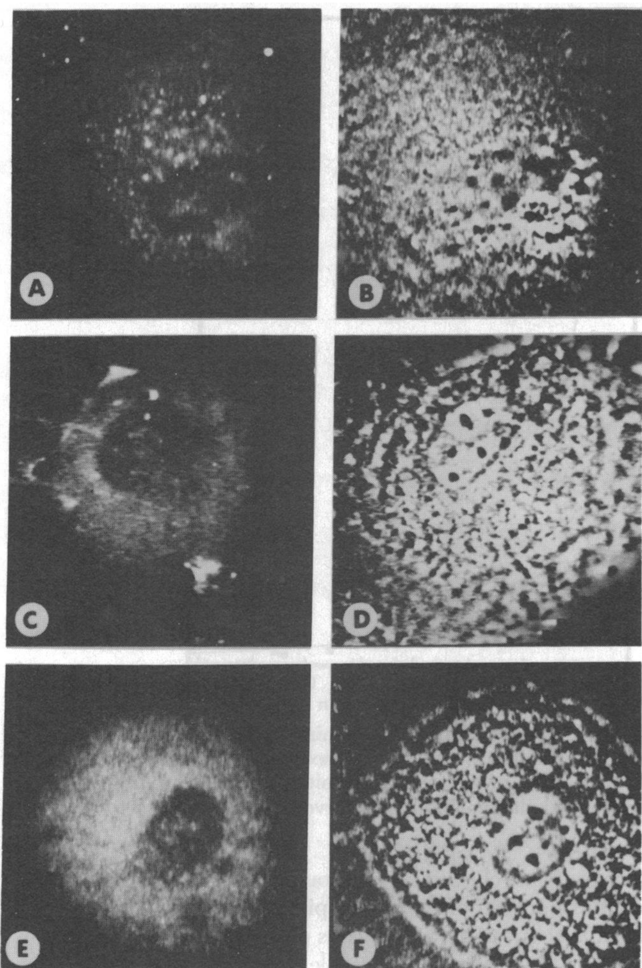

FIG. 1. Inhibition of EGF receptor clustering. (*A* and *B*) Fluorescence and phase-contrast photomicrographs of BALB/c 3T3 cells after a 20-min incubation with 10 nM rhodamine-labeled EGF at 37°C. (*C* and *D*) Same as *A* and *B* but 10 mM methylamine was added 20 min before the addition of rhodamine-labeled EGF. (*E* and *F*) Same as *A* and *B* but 0.7 mM bacitracin was added 20 min before the addition of rhodamine-labeled EGF. After the indicated incubations, cells were rinsed with serum-free medium and fixed with 2% formaldehyde. Fluorescence (*A, C, E*) and phase-contrast images of the same fields were recorded on videotape by using a silicon intensifier target television camera (19). (×680.)

50 mM methylamine inhibited 77%. CHO cell extracts possess higher levels of activity, so we used these cells to characterize the inhibition in more detail. The levels of transglutaminase activity, as determined by incorporation of putrescine into casein, vary widely among various cultured cell lines (23). Both methylamine and bacitracin inhibited transglutaminase activity in CHO cell extracts (Fig. 2*A*). Methylamine is well-known as a transglutaminase inhibitor (14); we are not aware of previous reports of transglutaminase inhibition by bacitracin.

Methylamine and bacitracin differed in the concentration dependence of their transglutaminase inhibition. Bacitracin inhibited about 40% of the transglutaminase activity at low concentrations ($K_i \approx 0.03$ mM), but higher concentrations produced no further inhibition. Methylamine was a weaker inhibitor, but approximately 65% of the enzyme activity was inhibited by 0.1 M methylamine. One explanation for these data is that there are two forms of transglutaminase activity in CHO cell extracts and they differ in their sensitivity to methylamine and bacitracin inhibition. Replotting the methylamine inhibition data as fractional inhibition/inhibitor concentration vs. fractional inhibition (Fig. 2*B*) emphasizes the two-component nature of the inhibition. Approximately 25% of the transglutaminase activity was inhibited by methylamine with $K_i \approx 0.2$ mM, and the rest of the activity was inhibited with $K_i \approx 20$ mM. The amount of transglutaminase inhibited with a low K_i varied between 25 and 50% of the total activity in different batches of CHO cells. When the bacitracin inhibition data were plotted this way (not shown), a single line was obtained. Recent studies (not shown) with several transglutaminase inhibitors showed an excellent correlation between the K_i and the concentration of the inhibitor that blocks clustering.

EGF Stimulation of DNA Synthesis. Because methylamine and bacitracin inhibit the clustering of occupied receptors, they provide a method for examination of the role of clustering over coated pits in hormone action. We examined the effect of methylamine and bacitracin on the EGF stimulation of DNA synthesis. In these experiments, quiescent BALB/c 3T3 cells were incubated with 1.5 nM EGF for 2.5 hr in the presence or absence of clustering inhibitors; [^{3}H]thymidine incorporation was measured 20–22 hr after the addition of EGF. As noted by Carpenter and Cohen (4), short incubations with EGF produced little stimulation of DNA synthesis. In various experiments, we observed approximately 1.5- to 5-fold stimulation of DNA synthesis in 2.5-hr incubations with EGF compared with 10- to 35-fold stimulation of DNA synthesis after 20-hr exposures to EGF (not shown). Three-hour incubations with 10 mM methylamine or 0.7 mM bacitracin also produced some stimulation of DNA synthesis (Fig. 3). In various experiments, the stimulation of [^{3}H]thymidine incorporation by methylamine or bacitracin ranged from 1.1- to 3-fold. Incubation with methylamine or bacitracin plus EGF resulted in a significant increase in DNA synthesis, and this increase was greater than the sum of the increases caused by EGF and the clustering inhibitor alone. In various experiments, the [^{3}H]thymidine incorporation in cells treated with EGF for 2.5 hr in the presence of 10 mM methylamine or 0.7 mM bacitracin was approximately 2- to 10-fold higher than the [^{3}H]thymidine incorporation in untreated cells. This stimulation was 2- to 7-fold greater than the stimulation produced by 2.5-hr incubations with EGF alone in parallel experiments. The stimulation of DNA synthesis by a 2.5-hr exposure to EGF in the presence of methylamine or bacitracin was approximately 30–50% of the stimulation produced by a 20-hr incubation with EGF.

Brief exposures to EGF were used in these experiments for two reasons. Because these brief exposures are suboptimal (4), they would allow potentiation of the hormone action to be expressed. Also, the brief exposures to the clustering inhibitors minimize any toxic side effects. After 20 hr in 10 mM methylamine, the cells were extensively vacuolized and many cells had detached from the dish. Both basal and EGF-stimulated DNA synthesis were greatly decreased under these conditions. Incubation with 1 mM methylamine for 3 or 20 hr had no effect on basal or EGF-stimulated DNA synthesis, and this concentration of methylamine did not significantly inhibit the clustering of rhodamine-EGF. Twenty-hour exposure to 0.7 mM bacitracin did not produce any obvious changes in the appearance of cells. Under these conditions, bacitracin did not potentiate EGF stimulation of DNA synthesis, and the stimulation of DNA synthesis by bacitracin alone (Fig. 3) was not observed. When BALB/c cells are grown for >20 hr in 0.7 mM bacitracin, their growth rate is slowed (not shown); the failure to observe stimulation of DNA synthesis in 20-hr exposures may be related to a slight toxicity of the bacitracin. We do not know why methylamine or bacitracin alone produced some stimulation of DNA synthesis. It is possible that they act by increasing the response of cells to growth factors in the culture medium.

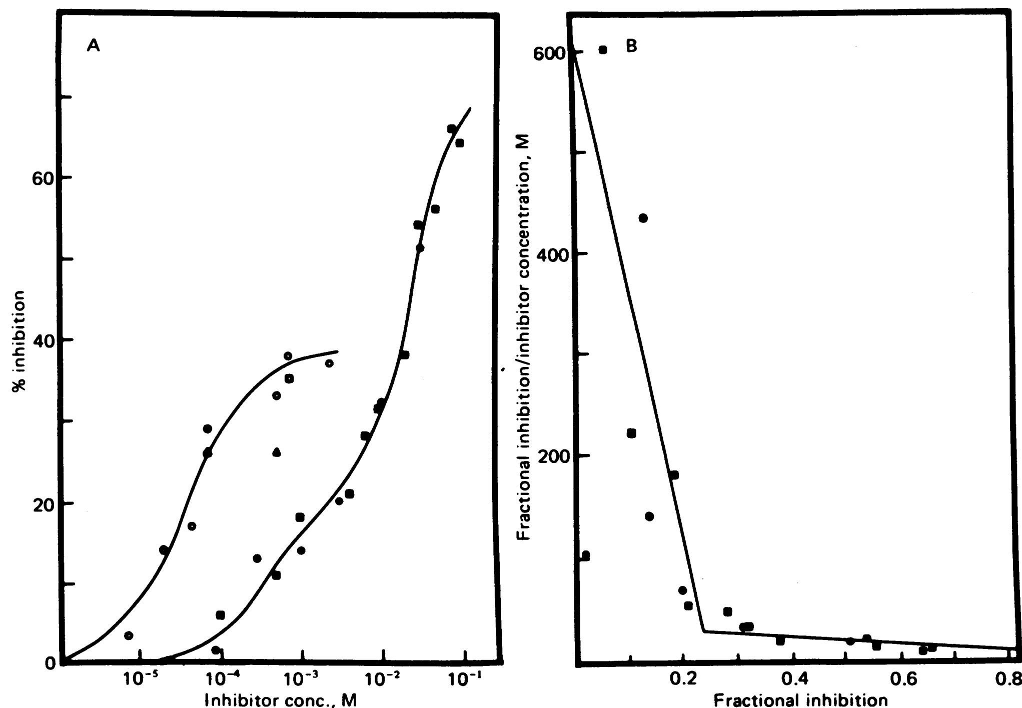

FIG. 2. (*A*) Inhibition of transglutaminase activity in CHO cell extracts by bacitracin (○, △, □) or methylamine (●, ■). The transglutaminase activity was determined by the incorporation of [^{3}H]putrescine into casein. The uninhibited activity in these experiments was 5–9.5 nmol/min per mg of protein^{-1}. Background activities, obtained in the absence of Ca^{2+}, were <10% of the total uninhibited values and were subtracted from all activities. The different symbols indicate separate experiments. (*B*) The methylamine inhibition curve was replotted as fractional inhibition/inhibitor concentration vs. fractional inhibition.

DISCUSSION

Role of Clustering in Hormone Action. If clustering of EGF over coated pits and the subsequent internalization in coated vesicles were required for hormone action, then methylamine and bacitracin should decrease the response to EGF. The observation that EGF stimulation of DNA synthesis is potentiated by methylamine and bacitracin indicates that extensive clustering and receptor-mediated internalization are probably not required for hormone action.

The bright fluorescent spots seen in Fig. 1*A* result from clustering over coated pits (7) and internalization in endocytic vesicles. These clusters contain roughly 100 EGF molecules (3), and the diameter of the coated pits is roughly 150 nm (8). It is possible that smaller aggregates of receptors form outside of coated pits and are not detectable by fluorescence microscopy. It has been suggested that such aggregates may be responsible for insulin (24) and EGF (25) action. We do not know if these small aggregates form in response to hormone binding or if their formation is affected by methylamine or bacitracin.

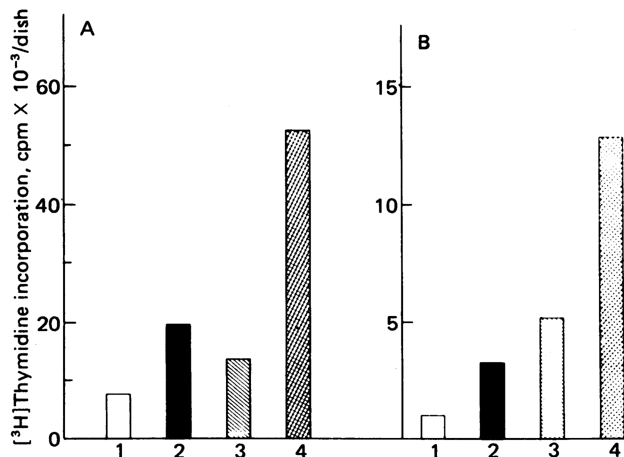

FIG. 3. Effect of 10 mM methylamine (*A*) or 0.7 mM bacitracin (*B*) on EGF stimulation (2.5-hr incubation) of DNA synthesis. Bars: 1, control; 2, test agent; 3, 1.5 nM EGF; 4, test agent plus EGF. Methylamine or bacitracin was added to the culture medium 30 min before the addition of EGF. Control dishes received no additions. [^{3}H]Thymidine incorporation was measured 20–22 hr after the addition of EGF.

The potentiation of EGF action by methylamine and bacitracin indicates that clustering of occupied receptors over coated pits and internalization in coated vesicles may be a mechanism to limit hormone action. It is likely that internalization results in hormone destruction (2). Most of the receptors also appear to be degraded (26), but internalized receptors may also be returned to the cell surface (13). Internalization and degradation of receptors may be responsible for the down-regulation of hormone receptors (27).

In cultured 3T3 fibroblasts, receptor-mediated endocytosis via coated pits appears to be the major pathway for internalization of α_2M, EGF, and insulin. Potentiation of EGF action by inhibitors of this pathway is consistent with the hypothesis that the cell surface is the site of EGF action, but other interpretations are also possible. Even if endocytosis via coated vesicles were completely inhibited, EGF would continue to be internalized by fluid-phase pinocytosis, and it is possible that EGF internalized by this mechanism is biologically active. Other lines of evidence suggest that persistent occupation of cell surface receptors is required for EGF action. Addition of antibodies to EGF up to 15 hr after addition of EGF to cells will decrease the stimulation of DNA synthesis (4). It has been reported that a small percentage of EGF bound to cells remains accessible to antibody binding up to 8 hr after EGF is removed from the medium and that this surface EGF is required for optimal hormone action (28).

We have observed that inhibition of insulin clustering did

not decrease the hormonal stimulation of α-aminoisobutyric acid uptake in freshly isolated hepatocytes (29). Previous studies with insulin linked to Sepharose beads to prevent endocytosis have also indicated that internalization is not required for insulin action, but the interpretation of these experiments is difficult because some highly active insulin-like material is released from the beads (reviewed in ref. 30). None of the experiments to date constitutes proof that EGF or insulin exerts its biological effects while on the cell surface, but several independent sets of experiments are consistent with that interpretation. It thus appears likely that a major role of polypeptide hormone internalization may be removal of occupied receptors from the cell surface.

Inhibition of Transglutaminase by Bacitracin and Methylamine. We have previously noted that primary alkylamines inhibit receptor clustering and are well-known inhibitors of transglutaminase (7); this, together with a Ca^{2+} requirement, indicated that transglutaminase might be involved in clustering. The crosslinks in proteins formed by transglutaminase have been found in membrane fractions of cultured cells (23). The observations reported here that the polypeptide antibiotic bacitracin inhibits *both* receptor clustering and cellular transglutaminase provides strong support for the hypothesis that transglutaminase-like activity is required for clustering. The molecular mechanism for bacitracin inhibition of transglutaminase has not yet been investigated. The ϵ-(β-asparaginyl)-lysine bond in the antibiotic (31) resembles the ϵ-(γ-glutamyl)lysine crosslink formed by guinea pig liver or plasma transglutaminase (14). A more detailed study of the correlation between transglutaminase inhibitors and clustering inhibitors will be presented elsewhere. Every transglutaminase inhibitor tested blocked receptor clustering, and every clustering inhibitor also inhibited transglutaminase. There is an excellent correlation ($P < 0.05$) between the K_i for transglutaminase and the concentration required to block clustering. The compounds tested include primary amines, lysine- or glutamine-containing peptides, bacitracin, a sulfonylurea (tolbutamide), and a site-specific irreversible inhibitor. The values of K_i for cellular transglutaminase ranged from 2.5 μM to 2 mM.

It is not clear why bacitracin inhibits only about 40% of the transglutaminase activity in CHO cell extracts, whereas methylamine inhibits all of the activity. One explanation for these data is that there are two forms of transglutaminase activity in CHO cells. Both forms can be inhibited by methylamine, but only one form is inhibited by bacitracin. Because bacitracin can completely inhibit clustering, we hypothesize that the form of transglutaminase that is inhibited by bacitracin is the form required for clustering. It is noteworthy that bacitracin inhibits transglutaminase at lower concentrations than methylamine (K_i, 0.03 mM vs. 0.2 mM), and it also completely inhibits receptor clustering at lower concentrations (0.7 mM vs. 10 mM).

Possible Cellular Regulation of Clustering. The selective clustering of occupied receptors provides an efficient mechanism for clearing occupied receptors from the cell surface without requiring extensive membrane turnover or recycling of unused receptors. It is interesting to note that cells possess mechanisms that might be used to regulate the rate of clustering. Transglutaminase levels in some cultured cell lines are increased at confluency and in response to cyclic AMP (ref. 32, P. Milhaud, personal communication). Also, the synthesis of putrescine, which could act as an endogenous inhibitor of transglutaminase crosslinking, is highly regulated by cells (33). Low levels of transglutaminase and high levels of putrescine, for example, might decrease the rate of hormone-receptor clustering and thus increase the responsiveness of cells to hormones. This type of mechanism may partially explain the observation that a 20-hr exposure to EGF gives a much greater mitogenic response than does short exposures. One of the early (4 hr) effects of EGF is the induction of ornithine decarboxylase which catalyzes the formation of putrescine (34). The increased levels of intracellular putrescine in response to the initial binding of EGF may decrease the rate of clustering and make the cells more responsive to the EGF which subsequently binds to the cell surface receptors.

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