INHIBITION BY BACITRACIN OF HIGH AFFINITY BINDING OF ^{125}I-α_2M TO PLASMA MEMBRANES

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1. Introduction

α_2-Macroglobulin (α_2M) is a large protein found in high amounts in plasma and other body fluids of many vertebrates. Many cell types have specific receptors for α_2M and rapidly internalize it after binding [1–11]. Fluorescence and electron microscopic data indicate that prior to binding α_2M, receptors are randomly distributed on the cell surface [9]. The α_2M-receptors randomly diffuse about in the cell membrane [12], bind α_2M, and become trapped in clusters in coated pits [7–10]. After accumulation in coated pits, α_2M rapidly enters the cells and is soon found in uncoated vesicles termed receptosomes [8].

The entry of α_2M is inhibited by a variety of compounds. Among these are primary amines such as methylamine and dansylcadaverine, and bacitracin [13–16]. Because these compounds are inhibitors of cellular transglutaminase activity, we have suggested that a transglutaminase-like enzyme participates in the internalization process [13,14,16].

The binding and internalization of α_2M by intact cultured fibroblasts have also been studied using ^{125}I-α_2M [11,15] at 4°C. Two classes of binding sites have been identified [11]. A high affinity class (K_d 0.2 nM) that is present in small numbers (10 000/cell) [11,15] and a low affinity class (K_d 100 nM) that is present in large numbers (600 000/cell) [11]. Transglutaminase inhibitors prevent binding to the high affinity class [11,15] but now the low affinity class [11]. We have suggested that the high affinity class represents receptor–ligand complexes clustered in coated pits because electron microscopic studies showed that methylamine prevented clustering of receptor–ligand complexes in these structures [13]. An alternative suggestion has been that the transglutaminase inhibitors act to prevent receptor recycling [15]. To distinguish between a direct effect on binding and an effect on receptor recycling we have studied the effect of bacitracin on α_2M binding to isolated plasma membranes in which receptor recycling cannot occur.

2. Experimental

For morphologic studies Swiss 3T3 cells were incubated at 4°C for 30 min with 30 μg α_2M/ml with or without 6 mg/ml bacitracin (Sigma). Then cells were fixed, sectioned and viewed by electron microscopy as in [13].

α_2M was prepared from whole human plasma as in [9,17]. α_2M was iodinated using either the chloramine-T procedure [6] at spec. act. 4×10^5 cpm/μg or the Bolton-Hunter reagent (New England Nuclear) at spec. act. 5×10^6 cpm/μg).

Purified plasma membranes were prepared from Balb/c 3T3 fibroblasts as in [18]. About 10% of the cellular protein was recovered in the plasma membrane fraction. The membrane pellet was diluted to 1 mg protein/ml in 10 mM Tris (pH 7.8), 1 mM EDTA, 1% Trasylol and frozen in aliquots. Before each experiment membranes were thawed, pelleted, and resuspended in 20 mM Hepes (pH 7.4) buffer. These plasma membranes contained both classes of binding sites for ^{125}I-α_2M.

Bacitracin was dissolved in H_2O at 100 mg/ml. For binding studies, 100 μl membrane suspension were preincubated for 20 min at 23°C with 1.5 mM $CaCl_2$, with or without bacitracin (2 mg/ml). Then BSA (to yield 2 mg/ml) and ^{125}I-α_2M were added. The total volume of each incubation was always 200 μl. Bind-

ing was carried out at 23°C and then the suspensions were cooled on ice. Membranes were pelleted at 4°C by centrifugation for 2 min in an Eppendorf centrifuge (model 5412). The pellet was resuspended at 4°C in Hepes (pH 7.4) containing 1.5 mM $CaCl_2$, centrifuged, and radioactivity in the pellet determined using an LKB mini-gammacounter.

Data from saturation analyses of binding were graphed by the Scatchard method [19] and analyzed by a computer program called SCATFIT [20]. This program employs a least square analysis and determines the number of classes of binding sites which best fit the data. The program also determines the parameters of each class of binding sites: the K_d and the capacity.

3. Results and discussion

3.1. *Inhibition of clustering of α_2M–receptor complexes in coated pits by bacitracin*

We have shown by electron microscopy that methylamine blocks clustering of α_2M–receptor complexes in coated pits. To be certain that bacitracin has the same effect, cells were incubated at 4°C with α_2M, labeled with peroxidase-anti-α_2M and visualized by electron microscopy. Peroxidase reaction product was seen concentrated in clathrin-coated pits (fig.1). Bacitracin reduced the appearance of peroxidase reaction product in coated pits (fig.1). Experiments were next carried out to determine if a direct effect of bacitracin could be detected in isolated plasma membranes.

3.2. *Direct inhibition of high affinity binding sites in plasma membranes*

Plasma membranes from Balb/c cells were incubated with 0.03 μg–3 mg α_2M/ml (fig.2). When the data were graphed by the Scatchard method [19] and analyzed by computer [20], a model consisting of 2 classes of binding sites best fit the data. One class had a high affinity (0.20 μg/ml or 0.24 nM) and a low capacity (0.4 μg/ml protein). The other class had a low affinity (60 μg/ml or 71 nM) and a high capacity (30 μg/mg protein). The affinities of these two classes of sites are similar to those using 4°C incubations of ^{125}I-α_2M with intact fibroblasts [11,15]. Both high and low affinity sites were recovered in 80% yield. In data to be presented elsewhere, we demonstrate that these binding sites have other properties in common with those of intact fibroblasts; binding is strongly dependent upon the presence of Ca^{2+}, and high affinity binding is selectively reduced by dansylcadaverine, another inhibitor of transglutaminase.

In the presence of 2 mg bacitracin/ml the high

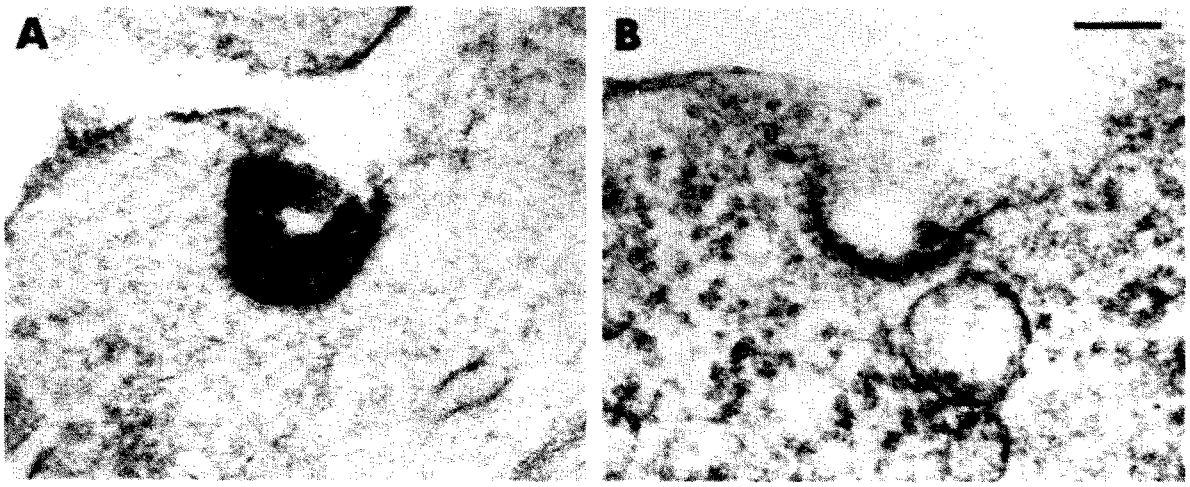

Fig.1. Swiss 3T3 fibroblasts were incubated with α_2M (30 μg/ml) at 4°C for 30 min in the absence (A) or presence of (B) bacitracin. Cells were then washed in the presence of bacitracin and incubated with peroxidase-labeled antibodies followed by fixation at 4°C. In (A) receptor–α_2M complexes are found clustered in coated pits. In (B) the α_2M does not cluster in coated pits. The small amounts of isolated peroxidase reaction product along the membrane is consistent with the diffuse distribution of α_2M on the majority of the cell membrane (Mag: × 108 000; bar = 0.1 μm).

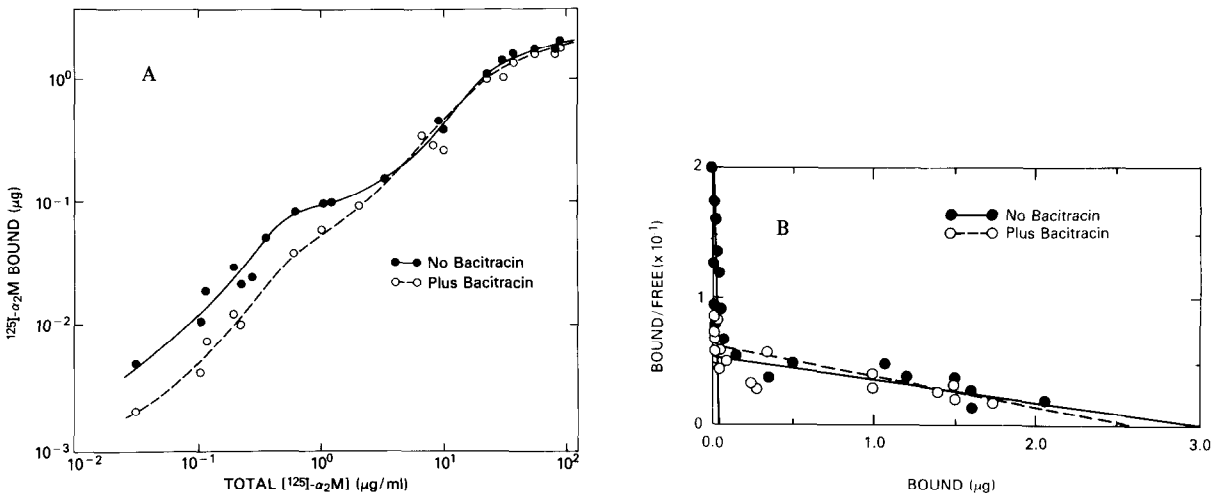

Fig.2. Concentration dependence of binding of ^{125}I-α_2M. Balb/c plasma membranes were incubated with various concentrations of ^{125}I-α_2M ± 3 mg/ml α_2M at 23°C for 40 min. Parallel incubations had been preincubated 10 min at 23°C with 2 mg/ml bacitracin. Binding data are plotted (A) as a saturation curve or (B) by the Scatchard method [19] and analyzed by computer [20] (see section 2): (○) bacitracin present; (●) bacitracin absent. Data are pooled from 2 separate expt.

affinity binding was reduced and primarily the low affinity class was detected (K_d = 40 μg/ml; capacity 25 μg/ml) protein (fig.2). We had observed using intact fibroblasts that high-affinity binding of ^{125}I-α_2M, as well as internalization, was much reduced by bacitracin [11]. In [15], binding of α_2M to high affinity receptors of intact normal human fibroblasts was blocked by another transglutaminase inhibitor, dansylcadaverine, and this effect was suggested to be due to an intracellular blockade of receptor 'recycling' [15]. It is not known if they failed to detect the majority of receptors, those with a lower affinity, or if normal human fibroblasts contain only high affinity sites.

Because recycling of receptors into the cell cannot occur in isolated membranes, this study shows that a transglutaminase inhibitor, bacitracin, directly blocks binding to high-affinity sites in plasma membranes of cultured fibroblasts. This supports a model in which a transglutaminase-like activity in the plasma membrane is required for conversion of low affinity receptors to high affinity receptors in the membrane. We suggest this occurs when α_2M receptor complexes cluster in coated pits. Our data does not support a model in which the inhibitors only affect receptor recycling.

The importance of this study is that for the first time a compound that inhibits internalization of α_2M has been shown to have a direct action on isolated plasma membranes. This study indicates that the transglutaminase-like activity postulated to be required for internalization is located in plasma membranes.

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