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Cellular Redistribution of β-Adrenergic Receptors in a Human Astrocytoma Cell Line: A Comparison With the Epidermal Growth Factor Receptor in Murine Fibroblasts

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The redistribution of β-adrenergic receptors (β-AR) during agonist-induced desensitization has been compared to the process of receptor-mediated endocytosis of epidermal growth factor (EGF) in human astrocytoma cells (1321N1). [^{125}I]EGF exhibited saturable binding to high affinity (K_D = 1–2 nM) receptor sites on intact 1321N1 cells. [^{125}I]EGF was found to internalize rapidly using an acid wash technique to remove surface bound hormone. Sucrose density gradient fractionation following exposure to EGF revealed a redistribution of EGF binding sites from high density (heavy peak) to low density (light peak) regions of the gradient. The light peak binding probably represents EGF in internalized vesicles formed during endocytosis. Low temperature (4°C) or the presence of the lectin concanavalin A (con A) inhibited this ligand-induced movement of EGF receptors. When cells were incubated simultaneously with EGF and the β-AR agonist isoproterenol, both receptors were found to co-migrate in the low density regions of sucrose gradients. No evidence of heterologous ligand-induced receptor endocytosis was found. These results suggest that the EGF receptors and β-AR are processed in parallel by 1321N1 cells.

Key words: EGF receptors, β-receptors, processing, sucrose gradients, concanavalin A

Agonist-induced desensitization of β-adrenergic (β-AR) function in human astrocytoma (1321N1) cells is a complex, multistep process leading first to the formation

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of an "uncoupled" form of the receptor [1,2]. Subcellular fractionation studies have shown that following the uncoupling reaction, β-ARs are redistributed from the plasma membrane into cytosolic fractions that contain markers for Golgi and endoplasmic reticulum [2,3]. The uncoupling and internalization reactions are rapidly ($t_{1/2}$ = 6 min) and completely reversed upon removal of the agonist. Continued exposure of 1321N1 cells to an agonist results in β-AR loss or down regulation [1,4]. At low cell density, down-regulated receptors can be recovered without protein synthesis. Such results suggest that in preconfluent cultures "lost" receptors, although undetectable by radioligands, are not degraded, but rather are sequestered intracellularly and can undergo functional reactivation upon removal of agonist. In contrast, if down regulation of β-AR is induced in postconfluent cultures, the lost receptors appear to be degraded and recovery of β-AR to normal levels upon removal of the agonist requires protein synthesis [4].

The process of receptor-mediated endocytosis has been described in many cell types and receptor systems [5-7]. For certain receptor systems, eg, low-density lipoprotein [5,8], asialoglycoprotein [9,10], and transferrin [11], internalization of the ligand appears to play a crucial role in metabolic responses subsequent to ligand binding. For other systems (insulin, epidermal growth factor, gonadotropin, luteinizing hormone-releasing hormone, lutropin, norepinephrine), the internalization of the ligand does not seem necessary for the initial biological response; indeed, a response can be elicited in the complete absence of hormone [12–19]. This suggests that a secondary (internal) signal is generated by the appropriate perturbations of the surface receptors. A large number of hormones are known to transmit their regulatory information through (positive or negative) alterations of intracellular cyclic AMP (cAMP) levels [20], and a good deal of research effort is currently focused on identifying second messengers for non-cAMP using systems (particularly growth-promoting hormones such as insulin, epidermal growth factor, and platelet-derived growth factor). For these types of hormone systems, receptor-mediated endocytosis has been proposed to function as a signal terminator and as a means of adaptively altering the cellular responsiveness to variable steady state levels of circulating hormone as the number of surface receptors reaches new steady states [21–23]. That receptor-mediated endocytosis performs such functions for the β-adrenergic receptor-adenylate cyclase system is supported by recent data [see 21,24 for review]. In addition, recent evidence has suggested that internalization of the epidermal growth factor-receptor complex may be necessary for the mitogenic response to occur [25–28].

The internalization of the EGF receptor (EGFR) has been well studied both biochemically and morphologically [for reviews, see 29–32]. In brief, EGF binds to relatively dispersed surface receptors, which are collected into coated pits, internalized via endocytotic vesicles, and then distributed to Golgi and lysosomal structures (where hormonal degradation occurs).

We have compared the binding and internalization of EGF receptors and β-AR to determine whether the changes in β-AR after binding of ligands are similar to those of the EGF receptor, which has been more completely characterized. In this report we demonstrate that EGF internalization occurs with similar characteristics in 1321N1 cells and in the Swiss 3T3 mouse fibroblast cell line, which has been more completely studied in this regard. Furthermore, we provide results indicating that catecholamine-induced internalization of β-AR and EGF-induced internalization of EGFR share certain features in 1321N1 cells.

MATERIALS AND METHODS

Cell Culture

Human astrocytoma cells (1321N1) were grown in Dulbecco's minimal essential medium (DMEM) plus 5% fetal calf serum and maintained as previously described [2,3]. Swiss 3T3 fibroblasts were grown in the same medium. Fibroblasts were used for 20–30 passages before being replaced with frozen stocks. All culture materials were obtained from sources previously cited [2,3].

Materials

Epidermal growth factor was purified by the method of Savage and Cohen [33] and iodinated by the chloramine T method [34]. Specific activities are given in the figure legends. Carrier-free $Na^{125}I$ was purchased from Amersham. Ultra-pure sucrose was from Schwarz/Mann and concanavalin A (con A) was obtained from Calbiochem. Pindolol, a generous gift from Sandoz (Basel, Switzerland), was iodinated by the chloramine T method, and [^{125}I] iodopindolol ([^{125}I]pin) was purified chromatographically as described [35]. Specific activity was 2.2 Ci/μmol. All other reagent grade chemicals were obtained from Sigma (St. Louis, MO).

Radioligand Binding Studies

All [^{125}I]EGF binding experiments were conducted on intact cells. Cultures were rinsed once with 20 mM HEPES-buffered Eagles minimum essential medium (HE) containing 0.1% bovine serum albumin (HEB, pH 7.4), then incubated with HEB containing [^{125}I]EGF (usually at 1–3 × 10^5 cpm/ml, 2–5 × 10^5 cpm/ng; see figure legends) at either 37°C or 4°C. At the end of the incubation, cultures were rinsed five times with 1 ml HEB at 4°C, then solubilized in 0.2 N NaOH for determination of cell-associated radioactivity. Nonspecific binding was determined in the presence of 2–5 μg/ml of unlabeled EGF and was subtracted from the total counts bound except where noted.

[^{125}I]pin binding was determined as previously described on crude membrane preparations [4]. Briefly, membranes (180 μl) were incubated with [^{125}I]pin (80–100,000 cpm, ~ 100 pM) in 2 mM $MgCl_2$ and 20 mM Tris buffer (pH 7.4) containing 1 mM ascorbate and 140 mM NaCl in a final volume of 0.25 ml. Incubations were for 60 min at 25°C. At the end of the incubation, the reaction mixture was diluted to 10 ml with 10 mM Tris (pH 7.4) containing 140 mM NaCl, vacuum-filtered (Schlicher and Schuell, #30), and rinsed with another 10 ml of buffer. Nonspecific binding was determined in the presence of 0.1 mM isoproterenol bitartrate. When sucrose gradient fractions were assayed, each fraction was first diluted 1:1 with 20 mM Tris (pH 7.4) containing 2 mM $MgCl_2$ and 140 mM NaCl. When gradient fractions from cells previously assayed for [^{125}I]EGF were to be assayed for [^{125}I]pin binding, additional parallel samples were incubated as just described but in the absence of [^{125}I)pin. The [^{125}I]EGF remaining on the filters was determined and subtracted from the total ^{125}I counts. This usually amounted to <5% of the total radioactivity.

Sucrose Gradient Centrifugation

Sucrose gradient fractionation of crude cellular lysates was done as previously described [3,36]. Three to five 150-mm tissue culture dishes of either 1321N1 cells or 3T3 fibroblasts were treated with 250 μg/ml con A in 10 ml HE or HEB for 20

min at 4°C. The con A treatment allowed for resolution of ligand binding sites across the gradients into two distinct peaks (see Results), presumably by maintaining the disrupted plasma membrane in open sheets rather than variably sized vesicles [see 3]. Cultures were then rinsed once with 10 ml of 2 mM ethylenediaminetetraacetic acid (EDTA) in 1 mM Tris (pH 7.4) at 4°C, and they were incubated for 10 min (3T3 cells) or 20 min (1321N1 cells) on ice in an additional 10 ml of the Tris-EDTA buffer. The hypotonically lysed cells were removed from the dish with a rubber policeman and loaded onto a continuous gradient of sucrose (30–60% or 15–40%, prepared with an ISCO model 570 gradient former) in 10 mM Tris (pH 7.4) at 4°C. The gradients were centrifuged for 60 min at 25,000 rpm (~115,000g) in a Beckman SW27 rotor, and fractions (1.8 ml) were subsequently collected from the top of the gradient with an ISCO model 568 fractionator. Under these conditions the membranes probably did not reach their equilibrium density. For subcellular fractionation of [^{125}I]EGF binding sites, cells were incubated with 10 ml HEB containing [^{125}I]EGF (10^6 cpm/40 ng EGF per dish) for various lengths of time at either 37°C or 4°C (see figure legends and Results). Cultures were then rinsed thrice with 10 ml HEB and twice with 10 ml HE. To the last rinse was added 5 ml of HE containing 750 μg/ml con A (final concentration of 250 μg/ml), and the cultures were processed as described above. Occasionally, the [^{125}I]EGF-containing fractions (maintained at 4°C) were filtered and rinsed as described above for the [^{125}I]pin binding assay to determine whether removal of free ligand would alter the gradient profile. When this was done the only change was a decrease in the radioactivity contained in the first 4 to 5 fractions, which represented the sample volume (cf, Fig. 5A,B). On the other hand, if the gradient fractions were allowed to incubate at 25°C for 60 min (see above) before filtration, virtually all the [^{125}I]EGF was lost to the filtrate. Thus, the [^{125}I]EGF did not appear to be trapped nonspecifically in vesicles. We therefore assume that the [^{125}I]EGF that entered the gradient was bound to its receptor. The data were not corrected for nonspecific binding.

Determination of Internalized [^{125}I]EGF

The amount of [^{125}I]EGF localized to the cell surface versus the cell interior was determined by a modification of the acid wash technique of Haigler et al [37]. Cells were incubated with [^{125}I]EGF at 4°C for 3–4 hr, rinsed five time with 1 ml HEB, and then incubated at 37°C. At the appropriate time, cultures were cooled on ice and the medium was removed and saved together with the subsequent rinse. Ice-cold 0.2 M acetic acid buffer (pH 4.0) in 0.25 M NaCl was added to each dish and incubated for 4–6 min on ice. The acid wash and subsequent rinse were combined and saved. The remaining (acid-stable, cell-associated) counts were removed from the dishes by solubilizing the cells in 0.2 N NaOH. The data were not corrected for nonspecific binding.

Determination of Protein Concentration and Cell Number

Protein concentration was determined by the method of Lowry et al [38] or Bradford [39] using bovine serum albumin as standard. Cell numbers were determined with a celloscope (Particle Data, Inc., Hialeah, FL) following incubation in 0.05% trypsin at 37°C and trituration to break up cell clumps.

RESULTS

A Comparison of EGF Binding to 1321N1 Astrocytoma Cells and 3T3 Fibroblasts

To determine if mouse 3T3 fibroblasts and 1321N1 astrocytoma cells behave similarly with respect to general characteristics of binding and internalization of EGF, the specific binding of [^{125}I]EGF to both cell types was measured at both 4°C and 37°C (data not shown). At 4°C, binding is presumably just to surface receptors [40], and both cell types display a typical binding isotherm with a plateau of binding being reached after 3–4 hr. At 37°C, [^{125}I]EGF binding also displays similar profiles for the two cell types, reaching a peak after 40–60 min for 3T3 cells and 60–90 min for 1321N1 cells, then declining with time to a steady state level. Comparable results have been reported by others [eg, 40,41].

Equilibrium binding assays in 1321N1 cells were done at 4°C to prevent internalization and degradation. The binding of [^{125}I]EGF to these cells was found to be a saturable process. Scatchard analysis [42] of the data revealed a single class of specific, high-affinity binding sites (2–3 × 10^4 receptors per cell) displaying a K_D of 1–2 nM (data not shown). We concluded that the interaction of EGF with 1321N1 cells was similar to that described in other systems [29–31].

Receptor-Mediated Internalization of EGF

Before performing subcellular fractionation experiments (see below), we compared the rate and extent of [^{125}I]EGF internalization in 1321N1 cells and 3T3 fibroblasts. Cells were prelabeled with [^{125}I]EGF at 4°C, washed, then incubated at 37°C. At the appropriate time, cells were cooled to 4°C, the medium was removed, and the cell monolayer was incubated in an acid buffer (see Materials and Methods) to remove surface bound hormone. The counts remaining with the cells (acid-stable, cell-associated) were then removed with NaOH and radioactivity was determined. The results show that EGF is cleared from the surface of both cell types very rapidly (Fig. 1) . No surface-bound (acid-labile) [^{125}I]EGF remained on 1321N1 cells after 30 min at 37°C ($t_{1/2} \sim$ 3–4 min) or on 3T3 cells after 10 min ($t_{1/2} \sim$ 1–2 min). The radioactivity lost from the surface appears, in part, in the medium (initially by dissociation of intact hormone from surface receptors, later owing to degraded hormone released from the cells; data not shown) and, in part, as cell-associated radioactivity (Fig. 1).

Since con A has been shown to inhibit the mobility of integral membrane proteins [42] and to inhibit agonist-induced redistribution of β-AR in 1321N1 cells [2], its effects on [^{125}I]EGF endocytosis were examined using the acid wash technique. Cultures were prelabeled at 4°C with [^{125}I]EGF as described above. (Note that in this as well as the sucrose gradient experiments [^{125}I]EGF was allowed to bind to cells before con A was applied). Following removal of unbound label, cultures were incubated for 20 min on ice in the presence or absence of 250 μg/ml con A. Cultures were rinsed once and incubated at 37°C. At the appropriate time the amount of radioactivity in the medium, the acid wash, and the cell monolayer was determined. Figure 2 shows the percentage of radioactivity remaining with the cells after the acid wash as a function of time at 37°C. When cells were treated with con A before warming, the percentage of cell-associated, acid-stable radioactivity was 2 to 3 times that found in control cells at time 0, and it remained constant or decreased slowly

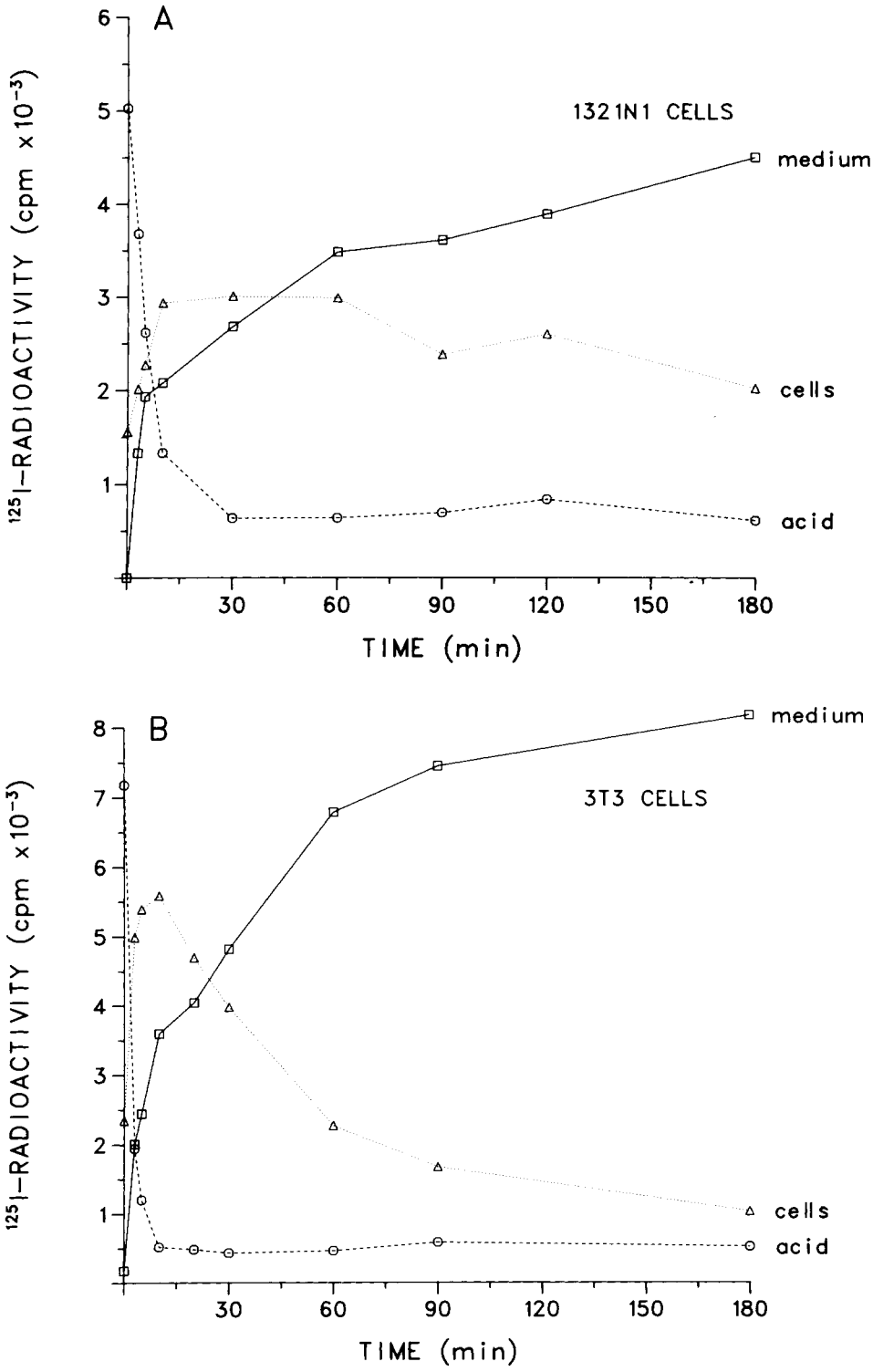

Fig. 1. Kinetics of internalization of [^{125}I]EGF by 1321N1 and 3T3 cells. Cells were prelabeled for 4 hr at 4°C with 4.8 × 10^5/2 ng/ml [^{125}I]EGF, rinsed, then incubated in 0.6 ml HBSA at 37°C. The amount of radioactivity present in the medium, the acid wash, and the cell monolayer was determined as described in Materials and Methods. A) 1321N1 cells; B) 3T3 cells.

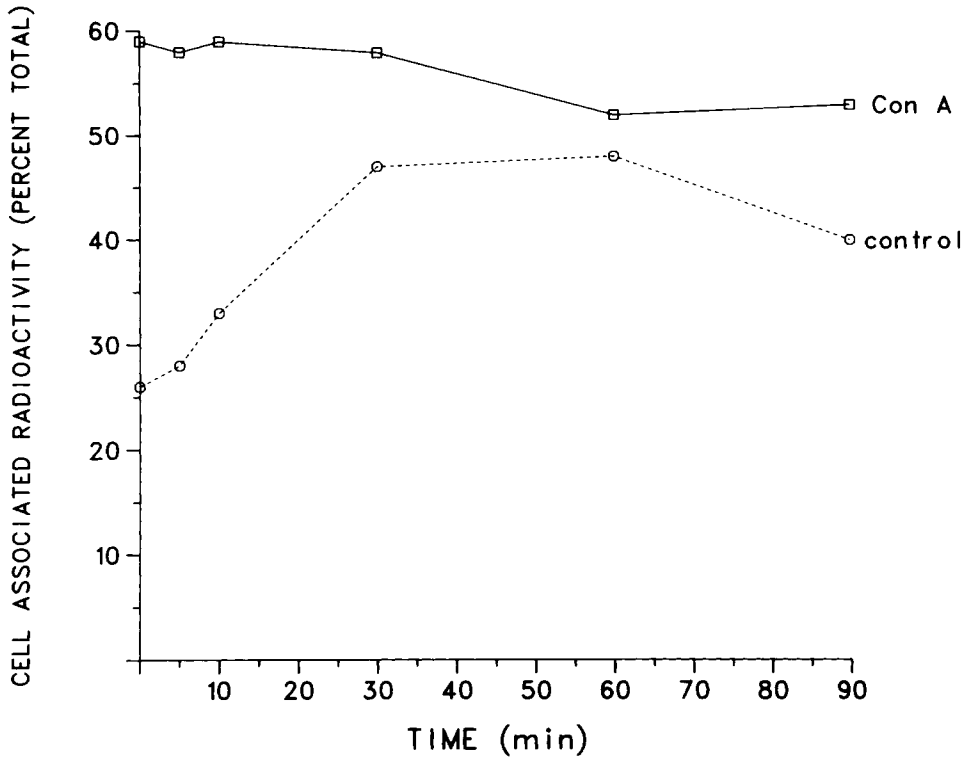

Fig. 2. Effect of conconavalin A on the amount of cell-associated radioactivity. All cultures were prelabeled for 3 hr at 4°C with 4 × 10^5cpm/4 ng/ml [^{125}I]EGF. Cultures were rinsed, incubated for 20 min at 4°C with (□) or without (○) 250 μg/ml con A, then incubated at 37°C for the indicated periods of time. Cultures were then treated as in Figure 1. The figure shows the amount of cell-associated radioactivity following an acid wash as a percent of the total (cell + acid wash + medium) radioactivity per dish.

over the course of the 37°C incubation. This result suggests that con A stabilizes the interaction of EGF with its receptor to disruption by low pH.

Similar experiments were done using the drug dansylcadaverine, which has been reported to inhibit the internalization of both EGF [37,44] and β-AR [45] receptors. No effect on [^{125}I]EGF distribution in 1321N1 or 3T3 cells was seen (data not shown).

Sucrose Density Gradient Fractionation of EGF Binding Sites: Co-Localization With β-AR

Previous reports have shown that particulate β-AR in 1321N1 astrocytoma [2,3,43] and rat C6 glioma [46] cells can be separated into low density (light peak) and high density (heavy peak) fractions on sucrose gradients. Incubation of cells with agonist induces an increase in light peak receptors and a concomitant decrease in heavy peak receptors. Plasma membrane markers all migrate in the heavy peak region. Subcellular fractionation experiments were conducted on cells incubated with [^{125}I]EGF in the presence or absence of a β-AR agonist (isoproterenol). Figure 3 shows the results of an experiment in which 1321N1 cells were incubated with

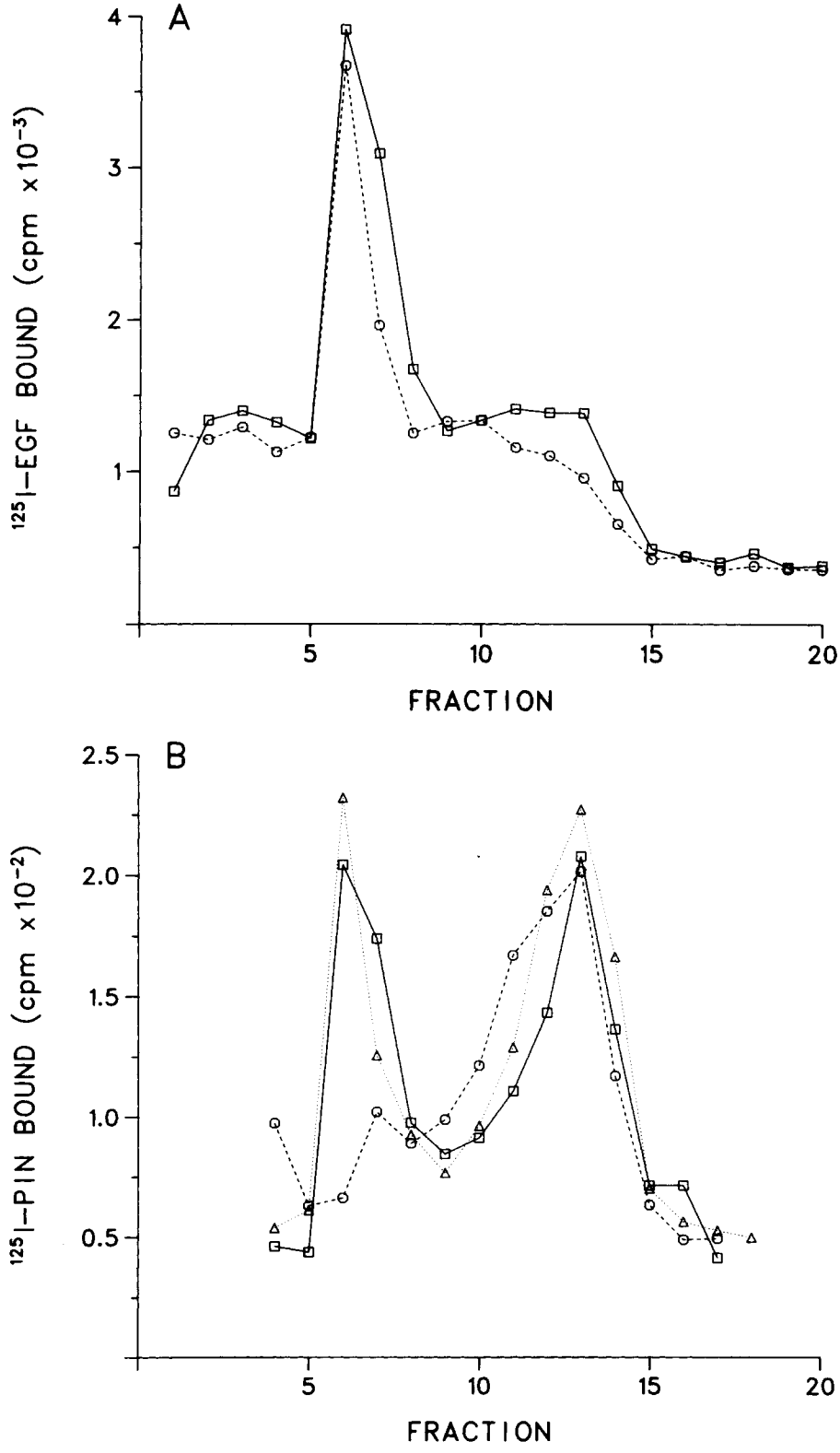
A
^{125}I-EGF BOUND (cpm x10^{-3})
4
3
2
1
5
10
15
20
FRACTION
B
^{125}I-PIN BOUND (cpm x10^{-2})
2.5
2.0
1.5
1.0
0.5
5
10
15
20
FRACTION

Fig. 3.

[^{125}I]EGF for 40 min at 37°C with or without 1 μM isoproterenol for the last 20 min of the incubation. A major portion of the [^{125}I]EGF binding sites (receptors) migrated near the top of the gradient (fractions 6,7; light peak), with a small amount of binding at fractions 11–13 (heavy peak) (Fig. 3A). This pattern was not altered by the presence of a β-AR agonist. An aliquot of each fraction was then assayed for [^{125}I]pin binding, and the results (after correcting for [^{125}I]EGF contribution to the radioactivity) are shown in Figure 3B. Those cells that were only incubated with [^{125}I]EGF show a typical control profile of [^{125}I]pin binding, ie, a small light peak and prominent heavy peak. Incubation with isoproterenol, with or without [^{125}I]EGF, induced a dramatic increase in the size of the light peak. Note that the [^{125}I]EGF and [^{125}I]pin binding occur in the same fractions.

The coincidence of EGF and β-AR receptor movement to the light peak fractions following exposure to their respective agonist was investigated further by conducting the centrifugal separation on a shallow sucrose density gradient. Lysates from cultures prelabeled with [^{125}I]EGF and incubated with 1 μM isoproterenol were run on 15–40% sucrose gradients. Under these conditions, most of the heavy peak sediments to the bottom, and the light peak is spread out over a third of the gradient (Fig. 4). The peaks of [^{125}I]EGF and [^{125}I]pin binding (measured on the same fractions; see Materials and Methods) migrated on the gradient at about 22–25% sucrose (range: 22–24% for [^{125}I]pin; 23–25% for [^{125}I]EGF). The peak of [^{125}I]pin binding was consistently found one fraction higher in the gradient than the [^{125}I]EGF binding, representing 1.5–2% lower sucrose concentration, or 5–10% of the total range of the gradient.

Receptor-mediated endocytosis is a temperature sensitive process [5,40]. Therefore, we examined the effect of temperature on the formation of the light-peak binding. 1321N1 cells were incubated in the continued presence of [^{125}I]EGF for 2.5 hr at 4°C or for 30 min at 37°C. Incubation at 4°C (Fig. 5A, closed circles) completely inhibited movement of receptor-bound ligand to the light peak, whereas cultures incubated at 37°C displayed a prominent light peak. 3T3 fibroblasts also were treated similarly and fractionated on sucrose gradients. As Figure 5B shows, not only do 3T3 cells show the same heavy and light peak distribution of [^{125}I]EGF binding sites as do 1321N1 cells, but they also show the same temperature sensitivity for light peak formation.

The effects of con A on light peak formation were tested. When 1321N1 cells (or 3T3 cells, data not shown)—prelabeled with [^{125}I]EGF at 4°C, rinsed, then

Fig. 3. Sucrose density gradient fractionation of [^{125}I]EGF and [^{125}I]pin binding sites in 1321N1 cells. Three to four 150-mm dishes were used for each gradient. Two sets of dishes were incubated with 10^5 cpm/4 ng/ml [^{125}I]EGF for 40 min at 37°C. One set received 1 μM isoproterenol for the last 20 min of the incubation. A third set received 1 μM isoproterenol for 20 min at 37°C, but no [^{125}I]EGF. At the end of the incubation, [^{125}I]EGF cultures were rinsed, then all dishes were processed for 30–60% sucrose gradients as described in Materials and Methods. After the [^{125}I]EGF-labeled fractions were counted, aliquots were removed, and [^{125}I]pin binding was determined in these fractions as well as the fractions from the cultures treated with isoproterenol alone. Correction for the residual [^{125}I]EGF was done. The gradient begins at fraction 5–6, the sample volume occupying fractions 1–4. A) [^{125}I]EGF binding; (□), [^{125}I]EGF + isoproterenol; (○), [^{125}I]EGF alone. Most of the binding sites are in the light peak (fractions 6–7), the heavy peak being contained in fractions 11–15. B, [^{125}I]pin binding; (□), [^{125}I]EGF + isoproterenol; (○), [^{125}I]EGF alone; (△), isoproterenol alone. Note that the presence or absence of EGF does not affect the distribution of [^{125}I]pin.

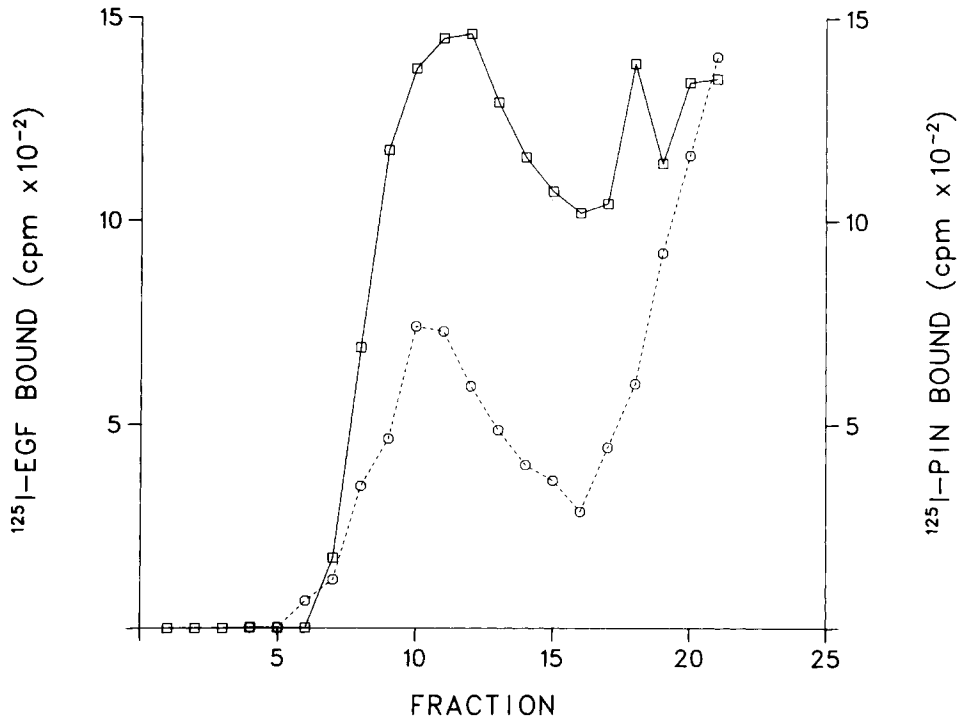

Fig. 4. Distribution of [^{125}I]EGF and [^{125}I]pin on 15–40% sucrose gradients. Cultures were treated as in Figure 3, except lysates were loaded onto 15–40% sucrose gradients. Sucrose gradient begins at fractions 5-6. (□), [^{125}I]EGF; (○), [^{125}I]pin.

incubated with con A at 4°C—were subsequently incubated at 37°C for 30 min, light peak formation was completely inhibited (Fig. 6). Cells treated with 500 μM dansylcadaverine produced the same profile of [^{125}I]EGF binding sits in sucrose gradients as did control cells (data not shown).

DISCUSSION

Different investigators using a variety of different experimental systems have proposed that catecholamines induce an internalized vesicular [2,3,47,48] or soluble [45] form of β-AR. The hypothesis used to explain this phenomenon [22] is based, however, on a model derived from both morphological and biochemical studies of polypeptide hormone receptor [49,50]. Little morphological data is available from β-AR experiments in support of this model. Studies with fluorescent-labeled antagonist ligands have not been enlightening in this regard [51], and to date the use of appropriately labeled β-AR antibodies has not been reported. We report here initial observations based on an alternate method for examining agonist-induced internalization of EGF receptors and β-AR.

We have examined in parallel the internalization of the EGF receptor and the β-AR in the astrocytoma cell line 1321N1, and compared it to the EGF receptor in murine fibroblasts, where receptor-mediated endocytosis of the EGF receptor com-

plex has been studied by both morphological and biochemical techniques [6,29–32,40,41]. Our results show that the binding of EGF to astrocytoma cells is a saturable process, and that unlabeled EGF competes for the binding of [^{125}I]EGF. [^{125}I]EGF binding to 1321N1 cells exhibits a dissociation constant of 1–2 nM, comparable to that found in other tissues [31]. EGF receptors have been reported to be present on a glial cell line [52] and have been detected on primary cultures of embryonic rat astrocytes [53, Wakshull, unpublished observations].

Using the acid wash technique of Haigler et al. [37] to distinguish between surface-bound and internalized [^{125}I]EGF, we have shown that surface-bound [^{125}I]EGF is rapidly internalized at 37°C, with the 3T3 cells clearing their surface of prebound EGF two to three time faster than the 1321N1 cells. Degraded hormone can be detected earlier in the mouse fibroblast cultures (15–30 min) than in the human astrocytoma cells (30–60 min; Wakshull, unpublished observations).

Con A has recently been reported to inhibit internalization of β-AR in 1321N1 cells [2] as well as to prevent homologous desensitization of protaglandin E_2 stimulation of cAMP synthesis in rat ovarian cells [54]. We therefore decided to examine the effects of this lectin on EGF receptor internalization using both the acid wash and sucrose gradient techniques. Although the results from the subcellular fractionation studies indicated that con A inhibits EGF receptor internalization, the acid wash experiments suggested that interaction of con A with the EGF receptor complex [55,56] involves more than immobilization of the receptors in intact cells, probably an increased binding of EGF, as described for human placenta membranes [56]. The enhanced level of acid-stable [^{125}I]EGF binding at all 37°C incubation times following con A treatment of prelabeled cells suggested that con A might alter the affinity of the EGF receptor for its ligand, making the complex stable to low pH disruption. A similar result has been reported for wheat germ agglutinin-nerve growth factor receptor interactions [57]. The nature of this con A effect is currently being investigated.

Agonist-induced movement of receptors into the light peak fraction of the sucrose density gradients is temperature sensitive. Thus, incubation at 4°C with [^{125}I]EGF resulted in labeled EGF appearing only in the heavy peak. All the [^{125}I]EGF could be removed by an acid wash of the intact cells (data not shown). However, if prelabeled cells were warmed to 37°C before fractionation, a dramatic increase in light-peak binding activity was seen. Not only is ligand-induced movement into the light peak temperature sensitive, but incubation of prelabeled cells with con A at 4°C completely inhibited this movement when cells were subsequently incubated at 37°C. Since internalization correlates with formation of the light peak, con A prevents ligand-induced EGF receptor internalization.

The results from the subcellular fractionation experiments demonstrate that the EGF receptor and β-AR display similar profiles on sucrose gradients. Although a one fraction (1–2% sucrose) difference in peak binding between the two receptors was detected, we cannot at this time attach much significance to this difference. Furthermore, movement of these receptors to the light peak region of the gradient is induced by interaction with the homologous agonist and is inhibited by low tempeature and con A. Although loss of EGF binding to rat adipocytes following β-AR stimulation has recently been reported [58], we found no evidence of heterologous down regulation of these two receptors in astrocytoma cells. Fractionation of both the 1321N1 and 3T3 cells resulted in coincident migratory behavior of [^{123}I]EGF binding sites in the sucrose density gradients.

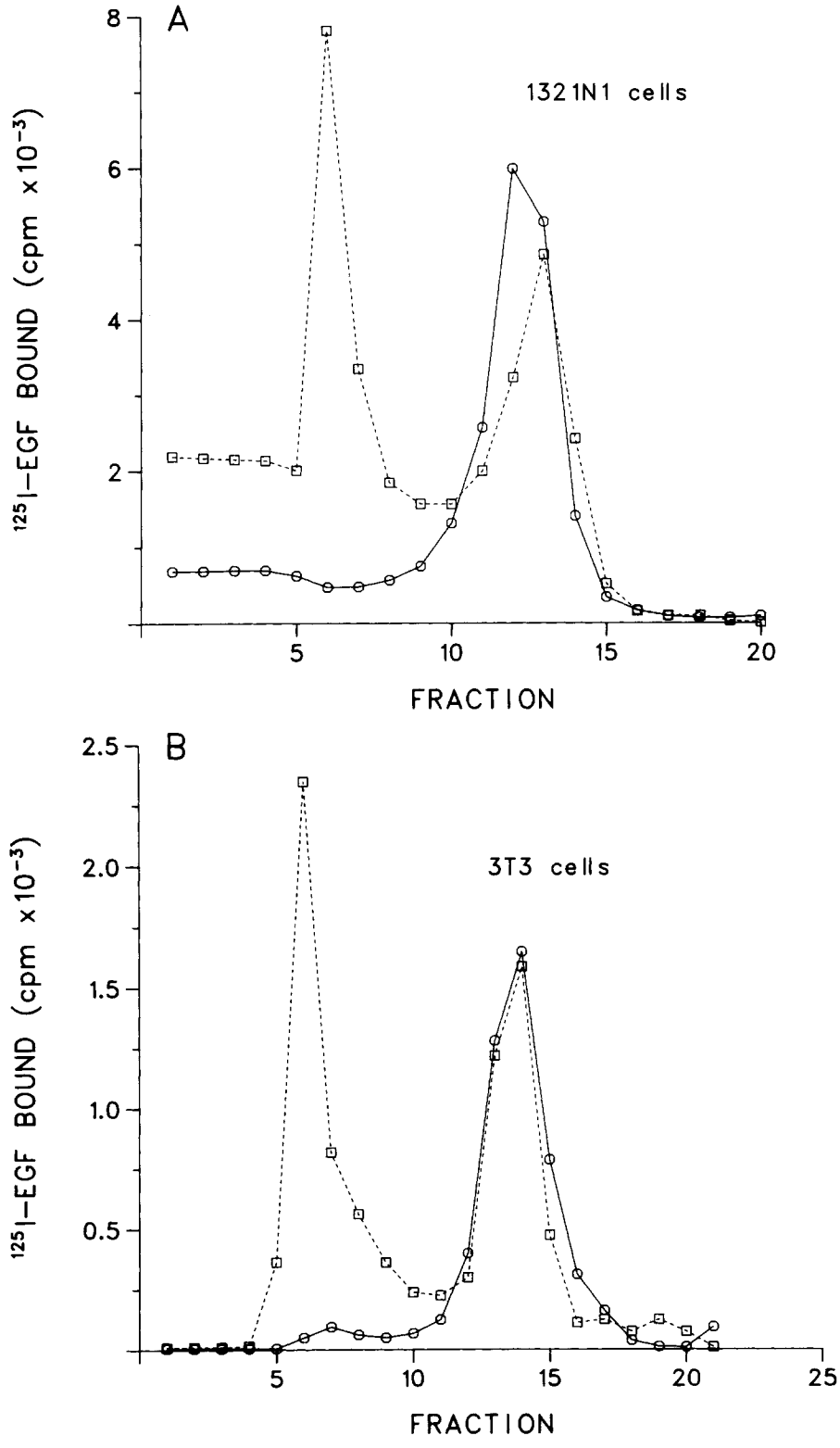
A
1321N1 cells
^{125}I-EGF BOUND (cpm x 10^{-3})
FRACTION
B
3T3 cells
^{125}I-EGF BOUND (cpm x 10^{-3})
FRACTION

Fig. 5.

Given the morphological data indicating the receptor-mediated endocytosis of EGF via the coated vesicle pathway in 3T3 cells, the data presented here suggest the use of this pathway by β_2-adrenergic receptors in 1321N1 cells following agonist binding. Although we cannot say from the experiments described whether the two receptors share the same vesicles during internalization, recent observations are compatible with this possibility [59–61]. It should be possible, using the techniques described in this report, to isolate the organelles involved in the removal of EGF-bound receptors from the cell surface, and to compare the properties of these receptors to their (ligand naive) cell surface counterparts.

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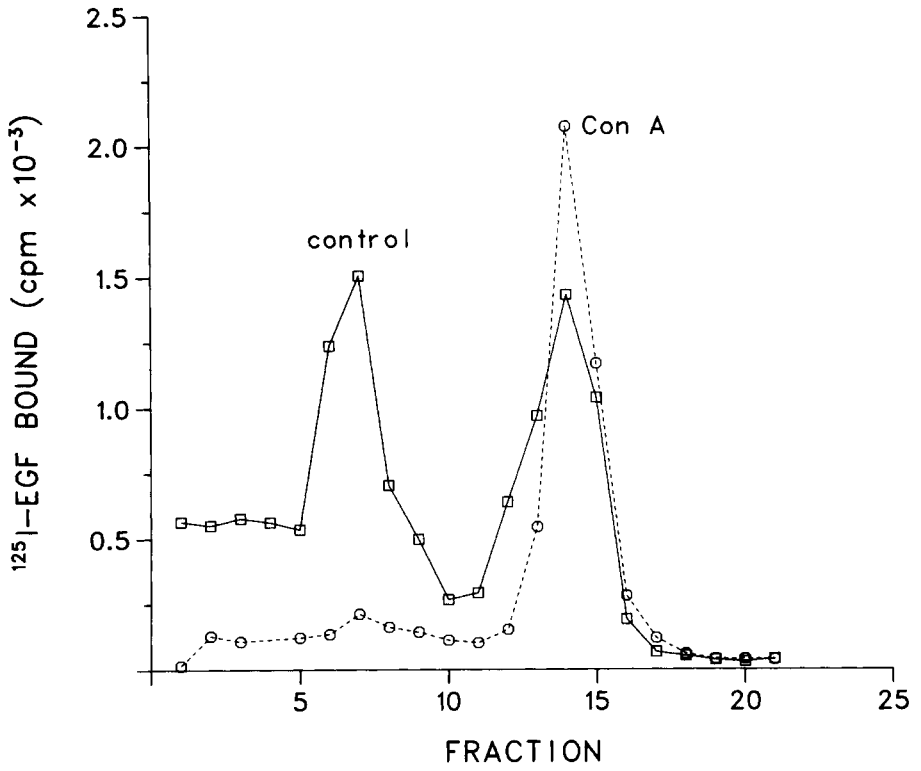

Fig. 6. Effect of conconavalin A on the distribution of [^{125}I]EGF in 30–60% sucrose density gradients. Cultures were prelabeled with 10^5 cpm/4 ng/ml [^{125}I]EGF for 3 hr at 4°C, rinsed, then incubated with (○) or without (□) 250 μg/ml con A for 20 min on ice. Cultures were shifted to 37°C for 30 min and then processed for sucrose gradients.

Fig. 5. Effect of temperature on [^{125}I]EGF distribution in 30–60% sucrose density gradients. Cultures were incubated with 10^5 cpm/4 ng/ml [^{125}I]EGF for 2.5 hr at 4°C or for 30 min at 37°C. Cultures were then processed for sucrose gradients as described in Materials and Methods. (□), 37°C; (○), 4°C. A) 1321N1 cells; B) 3T3 cells.

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