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Differential Effects of Antimycin A on Endocytosis and Exocytosis of Transferrin also Are Observed for Internalization and Externalization of β-Adrenergic Receptors

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SUMMARY

In many cells catecholamines induce a translocation of β-adrenergic receptors from the cell surface to intracellular vesicular sites. We have postulated that the translocation event is the result of ligand-induced endocytosis of the receptor, probably via clathrin-coated pits. Previously, we demonstrated that reduction of cellular ATP content with antimycin A completely blocked endocytosis of epidermal growth factor and translocation of β-adrenergic receptors in 1321N1 astrocytoma cells. However, the effect of reduction in ATP content on endocytosis remains controversial. In the present report, we demonstrate that reduction of ATP content to a level <5% of that in control cells is sufficient to prevent endocytosis of [^{125}I]iodotransferrin and translocation of β-adrenergic receptors. The further demonstration that reactions leading to the return of internalized transferrin or β-adrenergic receptors to the cell surface are blocked after relatively modest reductions in ATP content provides further evidence of the similarity in the processes subserving diacytosis of β-adrenergic receptors and transferrin. The differential requirement for ATP of the two arms of diacytosis provides the basis for an explanation of the controversy regarding a requirement for ATP in endocytosis via clathrin-coated pits.

In previous reports we (1–3) and others (4–6) have described the process of catecholamine-induced translocation of β-adrenergic receptors from the cell surface to what appear to be intracellular vesicular sites. In a series of studies comparing receptor-mediated endocytosis of EGF and ligand-induced translocation of β-adrenergic receptors in the same cells, both events exhibited the characteristics of endocytosis via clathrin-coated pits (7–9).

In one such study (9) we observed that reduction of cellular ATP had no apparent effect on ligand binding to the β-adrenergic receptor but completely prevented the ligand-induced receptor translocation reaction. Receptor-mediated endocytosis of EGF also was completely inhibited under the same conditions in the same cells. The results appeared to support the idea that β-adrenergic receptors are translocated by the same mechanism as are EGF receptors, namely, endocytosis via clathrin-coated pits. We further speculated that ATP was acutely required for endocytosis.

Although there is general agreement on a requirement for ATP for continuous uptake of transported ligands such as asialoglycoprotein (10), low density lipoprotein (11), and diferric transferrin (12, 13), most studies emphasize that ATP is required for recycling of receptors that are used repetitively to sustain ligand uptake. An initial internalization event (half-cycle) of pre-bound asialoglycoprotein (10), low density lipoprotein (11), or transferrin (12, 13) appeared not to be affected by reductions in ATP that completely inhibited continuous uptake of these molecules.

In contrast to such reports, we have presented evidence that even a first round of internalization of pre-bound EGF is prevented if ATP content is reduced by antimycin A and glucose-free medium (9). The inhibition was immediately reversed upon addition of glucose, which, even in the continued presence of antimycin A, caused a rapid rise in cellular ATP content. However, it is possible that events following ligand binding, but preceding the actual internalization of EGF, dictate the requirement for ATP. In this regard, it has been proposed that after binding of EGF, but before internalization, polymerization of EGF-receptor complexes occurs (14–16). If this step does not occur at 4° and requires ATP, then an alternative explanation for our previous results with EGF would be evident.

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ABBREVIATIONS: EGF, epidermal growth factor; DMEM, Dulbecco's modification of Eagle's medium; FBS, fetal bovine serum; CGP-12177, 4-(3-*tert*-butylamino-2-hydroxypropoxyethyl)-1-piperazineethanesulfonic acid; HEB, HEPES-buffered Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

In view of this potential complication, we have chosen to extend our analysis of the energy requirements for endocytosis by examining internalization of diferric transferrin as a model for the ligand-induced translocation of β-adrenergic receptors. Transferrin is carried into cells bound to a receptor that appears to cycle continuously as the aporeceptor (17, 18). No indications of requirements for receptor polymerization or of alternative pathways for internalization have been published regarding this system. Of further relevance are the observations that transferrin receptors are not appreciably degraded upon internalization and transferrin-receptor complexes do not dissociate within the cell (19, 20). Thus, receptor cycling can be readily monitored by appropriate measures of [^{125}I]iodotransferrin (21).

The results of the present study indicate that both endocytosis and exocytosis of transferrin are essentially completely inhibited by reduction of cellular ATP. However, the exocytosis process is more sensitive to reductions in ATP and is inhibited first after addition of antimycin A, which causes ATP levels to decline over a 20-min time span. Both processes are rapidly reinstated upon addition of glucose, even in the continued presence of antimycin A.

A comparison of the effect of antimycin A on internalization and recycling of β-adrenergic receptors and transferrin receptors in the same cell line supports the contention that β-adrenergic receptors are induced by catecholamines to undergo diacytosis by a pathway that is indistinguishable from that utilized by transferrin receptors.

Experimental Procedures

Materials. DMEM, Eagle's minimum essential medium, 0.25% trypsin, and FBS were obtained from GIBCO, culture dishes were from Nunc, and culture wells and culture flasks were from Corning. (−)-Isoproterenol bitartrate, DL-propranolol HCl, and antimycin A were obtained from Sigma, and ATP bioluminescence CLS was from Boehringer Mannheim. Pronase protease (*Streptomyces griseus*) was purchased from Calbiochem. The following drugs were gifts: (−)-cyanopindolol from Sandoz and CGP-12177 from M. Staehelin (Ciba Geigy, Basel, Switzerland). [^{125}I]Iodocyanopindolol was prepared by a modification of the method of Barovsky and Brooker (22). ^{125}I-labeled diferric transferrin was prepared according to the method of Karin and Minz (23). Specific activity was 4–6 × 10^8 cpm/nmol of transferrin.

Cell culture. Human astrocytoma cells (1321N1) were maintained in DMEM supplemented with 5% FBS, in an atmosphere of 92% air/8% CO_2 in a humidified incubator. The cells were fed on day 4 with fresh DMEM plus 5% FBS and were subcultured (1.8–2.0 × 10^6 cells) into a 75-cm^2 flask on day 7 by trypsinization (HEPES-buffered, pH 7.4, 0.05% trypsin solution, for 10 min at 37°). For experiments, cells were plated on 35-mm, 60-mm, or 100-mm tissue culture dishes (15,000 cells/cm^2) or six-well dishes (1.5 × 10^4 cells/well) and were used on day 4 unless otherwise indicated. Cell numbers were determined with a Coulter counter.

Binding of [^{125}I]iodocyanopindolol. To determine the total number of β-adrenergic receptors, lysates or membranes were incubated with 50 pM (10 × K_d) [^{125}I]iodocyanopindolol in 154 mM NaCl, 20 mM Tris, 5 mM $MgCl_2$, pH 7.4, in a total volume of 0.25 ml, for 2 hr at 30°. Nonspecific binding was determined as the binding occurring in the presence of 1 μM propranolol.

Internalization of β-adrenergic receptors. To determine internalized receptors, competition binding was performed with 10 pM [^{125}I]iodocyanopindolol and CGP-12177 at 30° for 2 hr. The highly hydrophilic ligand CGP-12177 has been shown to bind only to surface β-adrenergic receptors (24), whereas the lipophilic ligand cyanopindolol binds readily to both surface and internalized β-adrenergic receptors. 1321Nl cells were incubated with 1 μM (−)-isoproterenol for various times at 37°, chilled, washed with HEB, and lysed by hypotonic shock in 1 mM Tris, 2 mM EDTA, pH 7.4, for 20 min at 4°. Internalization of β-adrenergic receptors was measured by quantitation of the isoproterenol-induced shift to the right of the concentration-inhibition curve for the impermeant receptor antagonist CGP-12177 in competition with the lipophilic radioligand [^{125}I]iodocyanopindolol (24). The agonist-induced increase in [^{125}I]iodocyanopindolol binding (see Fig. 7) reflects the sequestration (internalization) of β-adrenergic receptors in sites not accessible to the membrane-impermeant competitor CGP-12177.

Internalization of β-adrenergic receptors also was determined by separation of internalized and cell surface forms of the receptor by centrifugation of cell lysates over gradients of sucrose (1, 25). Briefly, lysates were layered on top of a step gradient consisting of 3.2 ml of 15%, 4 ml of 38%, and 4 ml of 60% sucrose (w/v, in 20 mM Tris·HCl, pH 7.4) and were centrifuged for 30 min at 35,000 rpm in a Beckman SW 40 rotor. The interfaces were collected and diluted 1/1 with 154 mM NaCl, 20 mM Tris, 5 mM $MgCl_2$, pH 7.4, and β-adrenergic receptors were determined by binding of [^{125}I]iodocyanopindolol. The 15%/38% sucrose interface fraction contained the internalized receptors and the 38%/60% interface fraction contained the cell surface receptors.

Transferrin receptors. The binding of [^{125}I]iodotransferrin to intact cells in culture dishes and the measurement of internalized [^{125}I]iodotransferrin by Pronase treatment have been described by Karin and Minz (23). Briefly, the cells were incubated with 20 nM diferric [^{125}I]iodotransferrin (about 100,000 cpm/assay) in HEB or in an experimental solution for the indicated time at 37° or 4°. At the end of the incubation, the cells were washed three times with ice-cold HEB and incubated with 0.3% Pronase in HEB for 60 min on ice. The cells were completely detached from the dish by repeated pipetting and then they were transferred to a microfuge tube and centrifuged for 2 min in an Eppendorf microfuge. The radioactivity in both the supernatant and the cell pellet was determined. The radioactivity in the supernatant represented the surface-bound ligand and that in the cell pellet represented the intracellular or internalized [^{125}I]iodotransferrin.

The nonspecific binding determined at 4° in the presence of 1 μM transferrin was 6–8% of total binding. The data presented have not been corrected for nonspecific binding.

Protocol for ATP depletion. The protocol for depletion of ATP in 1321N1 cells has been described by Hertel *et al.* (9). After rinsing and 20-min incubation in glucose-free buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4), the cells were treated with antimycin A (30–300 nM) for 20 min at 37°. Then, radioligand and/or the test agents were added directly into the medium.

Determination of the intracellular ATP concentration. Cells were solubilized by addition of 1.5 ml of 0.14 N HNO_3 (70°) to each 60-mm dish. The incubation was continued for an additional 20 min at 37° to allow complete solubilization. The supernatants were collected and centrifuged for 10 min at 3000 × *g*. The pH of the supernatant was adjusted to pH 7.0 by addition of 0.6 N NaOH in 20 mM Tris.

The ATP content was determined using the ATP bioluminescence (CLS) assay by counting light emission in a Beckman scintillation counter with only one photomultiplier. CLS reagent (50 μl) was diluted in 3.5 ml of reaction buffer (20 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.18 mM dithiothreitol, 0.15 mM AMP, pH 7.0). Samples (100 μl) were added to 1 ml of this reaction mixture, and light emission was measured for 1 min. The signal was constant for at least 5 min, due to inhibition of the initial rate of the reaction by excess AMP. Under these conditions, a range of 5 pmol (10^5 cpm) to 40 pmol (10^6 cpm) of ATP could be determined.

Protein assay. The protein concentration of cell lysates was determined according to the method of Bradford (26), using bovine serum albumin as a standard.

Data presentation. The results in this report are presented as means ± standard errors unless otherwise indicated. Statistics were calculated using Student's *t* test, and the significance level was $p <$

0.05 (27). Most figures are representative of at least two similar experiments with duplicate or triplicate determinations.

Results

Transferrin receptors. Binding, endocytosis, and exocytosis of [^{125}I]iodotransferrin were characterized using human astrocytoma (1321N1) cells, according to procedures outlined by Ciechanover *et al.* (21). The time course of binding of [^{125}I]iodotransferrin at 4° and 37° is shown in Fig. 1, and the pattern of diacytosis of [^{125}I]iodotransferrin in 1321N1 cells is shown in the isotope chase experiment illustrated in Fig. 2. The results of such experiments demonstrated both qualitatively and quantitatively that 1321N1 cells bind and process transferrin in a manner comparable to that of the HepG2 cells studied by Ciechanover and co-workers.

As reported in 1986 (9), we also found that treatment of 1321N1 cells with antimycin A (30–300 nM) in a glucose-free medium resulted in a marked reduction in cellular ATP content. The ATP content of control cells was reduced from 3–4 mM to <5% of this range with a $t_{1/2}$ of 4 min (data not shown). Incubation of 1321N1 cells with 300 nM antimycin A for 20 min at 37° was the standard ATP reduction protocol. Addition of glucose (2 mg/ml) resulted in a rapid accumulation of cellular ATP to near-control levels within 10 min (data not shown; see Ref. 9).

In Fig. 3, the marked inhibition by antimycin A of the internalization of [^{125}I]iodotransferrin is illustrated. Addition of glucose after a 20- or 40-min exposure to antimycin A caused

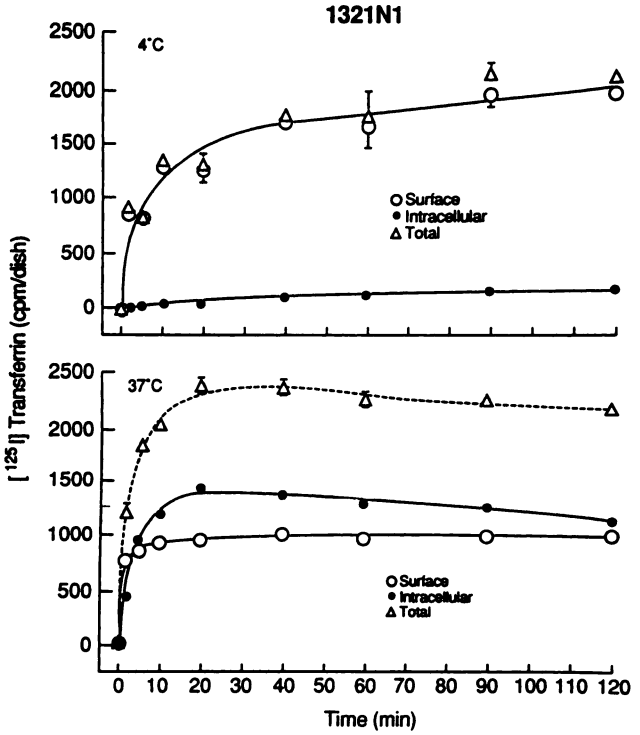

Fig. 1. Time course of cell surface binding and internalization of [^{125}I]iodotransferrin. Cells grown in 35-mm dishes were incubated with 20 nM [^{125}I]iodotransferrin at either 4° or 37° for the times indicated. The cells were washed at ice temperature and treated with Pronase as described in Experimental Procedures. Vertical bars represent the range of duplicate values when the range exceeds the dimension of the symbol. The data represent values from a single experiment, which was conducted two times with similar results.

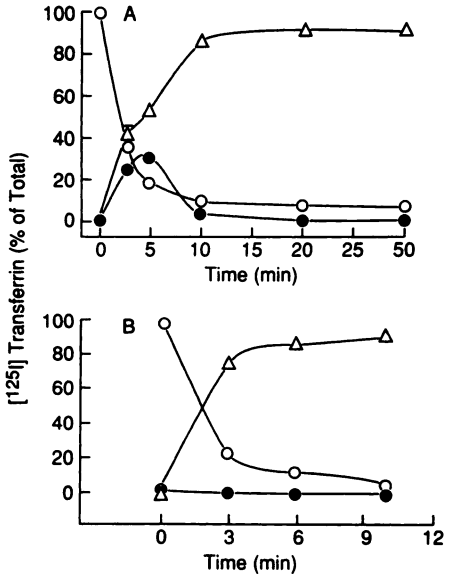

Fig. 2. Diacytosis of [^{125}I]iodotransferrin before and after reduction of cellular ATP content with antimycin A. Cells grown in 35-mm dishes were incubated with 20 nM [^{125}I]iodotransferrin for 60 min at 4°. After washing at 4° to remove free ligand, the cells were incubated with prewarmed HEB containing 200 nM transferrin at 37° for the times indicated. The medium was aspirated and its radioactivity was assessed (△), and then the cells were chilled on ice and treated with Pronase to release surface-bound [^{125}I]iodotransferrin (○). The cell pellet obtained by centrifugation after Pronase treatment contained the internalized radioligand (●). The data are expressed as a percentage of total radioactivity (medium plus surface plus intracellular). A, Control; B, treated with 300 nM antimycin A for 20 min at 37° before the binding reaction at 4°. The antimycin A was included in the medium throughout the experiment. The data represent values from a single experiment, which was conducted three times with similar results.

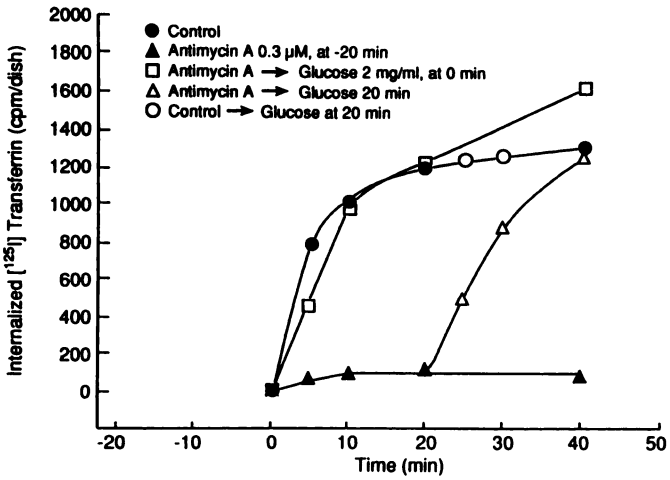

Fig. 3. Reversal by glucose of the inhibition by antimycin A of internalization of transferrin. Cells were incubated (▲, □, △) or not (●, ○) with antimycin A (0.3 μM) at 37° (−20 to 0 min). At 0 min [^{125}I]iodotransferrin (20 nM) was added to control (untreated) cells and the time course of internalization was determined by Pronase treatment as described. At 20 min glucose (2 mg/ml) was added to the control cells (○). [^{125}I]Iodotransferrin also was added after 20 min of treatment with antimycin A (at 0 min) in the presence (□) or absence (▲) of glucose. At 20 min glucose was added to the cells exposed to antimycin A (△). The data represent values from a single experiment, which was conducted two times with similar results.

rapid reversal of the inhibition of internalizaion. Addition of [^{125}I]iodotransferrin and glucose simultaneouly to cells treated with antimycin A for 20 min resulted in a slightly reduced rate of intracellular accumulation of [^{125}I]iodotransferrin, compared with the rate in cells not treated with antimycin A. This result probably reflects the fact that ATP levels are not restored instantaneously; the reaction exhibits a $t_{1/2}$ of about 3 min (9), which is comparable to the $t_{1/2}$ for endocytosis of [^{125}I]iodotransferrin (21). This experiment does not distinguish between inhibition of [^{125}I]iodotransferrin binding and inhibition of internalization.

To address the question of a requirement for ATP for the endocytic event *per se*, we carried out the experiment shown in Fig. 2B. Cells were treated with antimycin A for 20 min at 37° and then exposed at 4° to [^{125}I]iodotransferrin in the continued presence of antimycin A. It has been established by others (21) and confirmed by us (Fig. 1) that endocytosis of [^{125}I]iodotransferrin is blocked essentially completely at 4°. Under these conditions, the extent of surface binding was reduced by about 50% in antimycin-treated cells, compared with untreated control cells (see below). When the cells were washed free of unbound [^{125}I]iodotransferrin and the temperature was raised to 37°, no endocytosis occurred in the ATP-depleted cells (Fig. 2B). The results indicate that a step between ligand binding and the appearance of [^{125}I]iodotransferrin in a protease-resistant (intracellular) location is inhibited by antimycin A.

Because it was possible that previous reports that reduction in ATP content did not block a single round of endocytosis of transferrin (11–13) were based on inadequate reduction in ATP content, we carried out a comparison of our experimental protocol and that of Podbilewicz and Mellman (13). Thus, the relation between temperature and the capacity of antimycin A to block [^{125}I]iodotransferrin internalization was examined, as shown in Fig. 4. The experiment was designed to compare the kinetics and extent of transferrin uptake in control cells and in cells that had been pretreated with antimycin A for either 20 min at 37° or 60 min at 4°. In each case, binding of [^{125}I]iodotransferrin was carried out at 4° for 60 min, followed by washing and addition of excess unlabeled transferrin. The results indicate that, as expected, treatment of cells with antimycin A for 20 min at 37° completely prevented internalization. This treatment reduced ATP content to <5% of controls and reduced cell surface binding sites for [^{125}I]iodotransferrin by about 50% (data not shown). Prior exposure of cells to antimycin A for 60 min at 4° reduced ATP content by <50% and reduced cell surface binding sites by only 20% (data not shown). When cells treated in this latter manner were then incubated at 37°, internalization of [^{125}I]iodotransferrin occurred at about one half the rate of controls (Fig. 4). Interestingly, with prolonged incubation internalized radioligand was not lost from the cell under these conditions. We conclude from the results that treatment with antimycin A for 60 min at 4° is not sufficient to reduce ATP to levels that completely inhibit endocytosis. Thus, when the temperature was raised to 37° internalization of the bound [^{125}I]iodotransferrin began. Because the cells were in the continuing presence of antimycin A, ATP levels declined further and endocytosis was eventually blocked. Because the internalized [^{125}I]iodotransferrin was maintained even in the presence of excess unlabeled transferrin, it appears that exocytosis was blocked as well. This is in contrast to the normal pattern of diacytosis observed in controls (Figs. 2A and 4).

When 1321N1 cells were incubated at 37° with a fixed concentration of [^{125}I]iodotransferrin, label accumulated in both intracellular and cell surface sites at fixed proportions that were maintained for at least 60 min (Fig. 1, *lower*, and Fig. 5). When antimycin A was added to cells exhibiting this steady distribution, the ratio changed over 20 min from about 58 internal:42 surface sites to about 75 internal:25 surface sites and was stable thereafter in the continued presence of antimycin A (Fig. 5). Upon addition of glucose to such antimycin A-treated cells, the ratio rapidly returned toward control values.

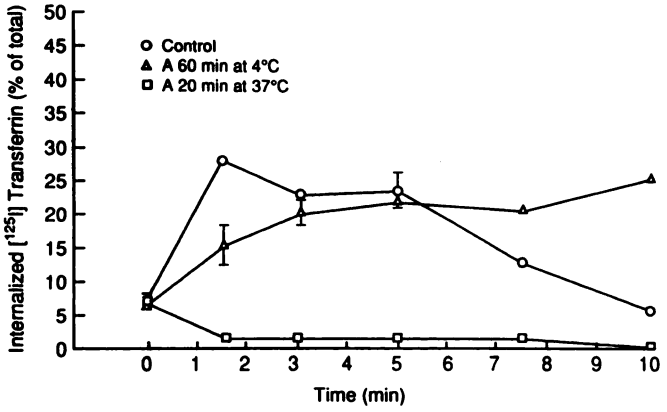

Fig. 4. Effect of temperature on the inhibition by antimycin A of transferrin internalization. Cells were incubated with 0.3 μM antimycin A for either 20 min at 37° (□) or 60 min at 4° (△) or were not treated (○). The cells were then chilled to 4° and exposed to [^{125}I]iodotransferrin for 60 min. The cells were washed to remove free [^{125}I]iodotransferrin and were incubated at 37° in the presence of 200 nM transferrin in the continued presence (□, △) or absence (○) of antimycin A. Data represent the percentage of total bound [^{125}I]iodotransferrin associated with the cells after Pronase treatment. *Vertical bars*, range of duplicate values when the range exceeds the dimension of the *symbol*. The data represent values from a single experiment, which was conducted two times with similar results.

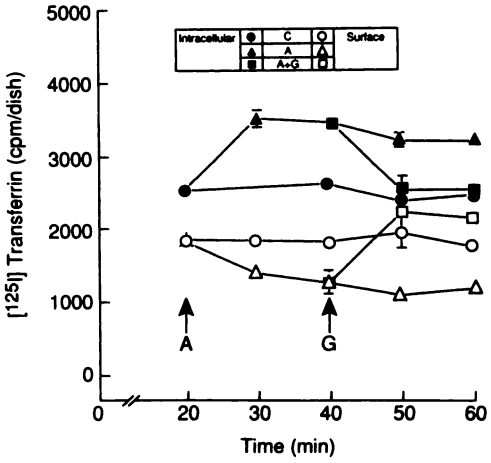

Fig. 5. Effect of antimycin A on the cellular distribution of transferrin receptors. Cells were incubated with 20 nM [^{125}I]iodotransferrin for 60 min at 37° (●, ○). Some cells also received antimycin A (*A*) (0.3 μM) after 20 min of incubation with [^{125}I]iodotransferrin (▲, ■, △, □). To some of the antimycin A-treated cells glucose (*G*) (2 mg/ml) was added after 20 min (■, □). At the times indicated, cells were chilled to 4° and surface and intracellular [^{125}I]iodotransferrin was determined by the Pronase procedure, as described in Experimental Procedures. The data represent values from a single experiment, which was conducted two times with similar results.

The results from experiments such as that shown in Fig. 5 suggest that the initial effect of antimycin A is on exocytosis of internalized [^{125}I]iodotransferrin, with endocytosis being blocked later as ATP content further declines. The results shown in Fig. 6 confirm the conclusion that exocytosis is blocked by antimycin A. It is clear that internalized [^{125}I]iodotransferrin does not exit the cell after ATP content is reduced; however, upon addition of glucose the internalized ligand rapidly leaves the cell.

Interestingly, the glucose-released [^{125}I]iodotransferrin does not simply appear in the medium, as would be expected if the process of exocytosis were followed by rapid dissociation of [^{125}I]iodotransferrin from the receptor. The previously internalized radioligand was found to transiently associate with the cell surface before appearing in the medium (Fig. 6). We return to this observation in the Discussion.

β-Adrenergic receptors. In the past our research has dealt primarily with the phenomenon of catecholamine-induced desensitization and down-regulation of the β-adrenergic receptor. We (1–3) and others (4–6) have shown that catecholamines induce the translocation of β-adrenergic receptors from the plasma membrane apparently to an intracellular membrane localization. We previously put forth the argument, based on biochemical data, favoring endocytosis via clathrin-coated pits as the mechanism underlying β-adrenergic receptor translocation (28, 29). Only recently, however, has convincing morphological data appeared to support such a model (30).

In Fig. 7 the effect of antimycin A on the disposition of β-adrenergic receptors in catecholamine-treated cells is illustrated. Previous studies have established that in untreated 1321N1 cells approximately 90% of cellular β-adrenergic receptors are at the cell surface and exposure to isoproterenol (0.1–1.0 μM) for 20 min results in a redistribution such that about

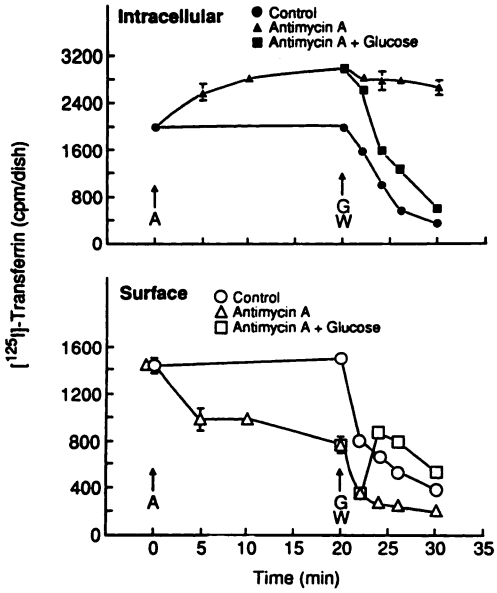

Fig. 6. Effect of antimycin A on exocytosis of internalized [^{125}I]iodotransferrin. Cells were incubated in the presence of 20 nM [^{125}I]iodotransferrin for 20 min at 37°. At this point (in time 0 min) some cells received antimycin A (*A*) (0.3 μM) (▲, ■, △, □). At the 20-min time point, cells were washed free of [^{125}I]iodotransferrin with HEB alone (▲, △) or with HEB containing glucose (*G*) (2 mg/ml (●, ■, ○, □). *Vertical bars*, range of duplicate values when the range exceeds the dimensions of the *symbol*. The data represent values from a single experiment, which was conducted two times with similar results. G, glucose; W, washed.

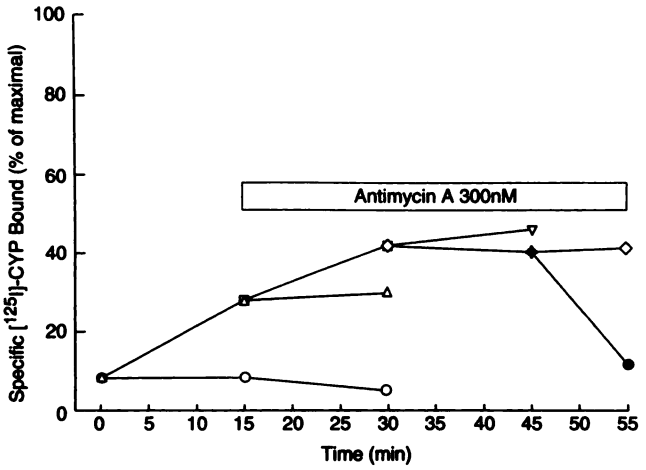

Fig. 7. Effect of antimycin A on the cellular distribution of β-adrenergic receptors in the presence of isoproterenol. *Ordinate* values are proportional to the percentage of β-adrenergic receptor internalized (see Experimental Procedures). Cells were incubated at 37° in the absence (○) or presence of 1.0 μM isoproterenol. After 15 min some of the isoproterenol-treated cells received antimycin A (0.3 μM) and some did not (△). After exposure to antimycin A for 15 min some cells were washed with the same medium (▽) and some were washed free of isoproterenol (●, ◇). After an additional 15-min incubation in the absence of isoproterenol, some cells received glucose (2 mg/ml) (●) and some did not (◇). The data represent values from a single experiment, which was conducted three times with similar results. *CYP*, cyanopindolol.

50–70% of receptors remain on the cell surface and 30–50% reside in a cytosolic compartment. The reaction is completely reversible upon removal of isoproterenol and the receptors can be shown (31) to recycle in the continued presence of isoproterenol ($t_{½in}$, approximately 2 min; $t_{½out}$, approximately 4 min). As shown in Fig. 7, addition of isoproterenol to the cells increased the content of receptor in the internalized form, in this experiment from 8 to 32%. Addition of antimycin A caused an additional increase in the internalized form. Removal of isoproterenol but continuation of exposure to antimycin A did not result in redistribution of the receptors. However, removal of isoproterenol plus addition of glucose resulted in rapid redistribution of receptors to control values.

Discussion

Results presented in this study bear on two issues. The first is the role of ATP in the process of receptor-mediated endocytosis via clathrin-coated pits. The second is the mechanism subserving catecholamine-induced β-adrenergic receptor internalization and externalization.

Our previous studies (9) showed that reduction of the ATP content of 1321N1 cells by antimycin A had little effect on the magnitude of binding of EGF to cell surface receptors but completely blocked EGF internalization. We used internalization protocols similar to those in this study to demonstrate that antimycin A blocked EGF uptake at a step distal to receptor binding and proximal to receptor recycling.

Our present studies indicate that reduction of the ATP content of 1321N1 cells to <5% of normal levels prevents endocytosis of transferrin. The same observation now holds for transferrin, EGF, and β-adrenergic receptors in these cells. The obvious conclusion is that ATP is required for endocytosis, either directly or indirectly. The experimental design and the nature of the receptor systems studied preclude the interpre-

tation that inhibition of endocytosis is only a consequence of inhibition of receptor exocytosis, as has been proposed by others (10–13).

Even though it is clear that endocytosis of transferrin is blocked by reduction of the ATP content in 1321N1 cells, when we examined the effect of antimycin A under steady state conditions the ratio of internalized to surface transferrin increased. Further study suggested that this apparently anomalous finding was the result of preferential inhibition of exocytosis of transferrin during the early stages of the fall in cellular ATP. Thus, if exocytosis requires substantially higher concentrations of ATP than does endocytosis, then incomplete reduction in ATP levels could lead to the situation in which sustained ligand uptake is blocked but endocytosis of pre-bound ligand still occurs. The difference between our results and those of others (10–13) could be explained if the level of ATP required for endocytosis varies in the different cell types examined. For example, Podbilewicz and Mellman (13) found that ATP levels in MDCK cells were reduced to 10% of control after 60 min at 4° in the presence of transferrin, NaCN, and 2-deoxyglucose. When the cells were washed free of unbound transferrin and the temperature was raised to 37°, the previously bound transferrin was internalized. We used essentially the same protocol (Fig. 4) and observed that 1321N1 cells also internalized transferrin when the temperature was raised to 37°. However, when ATP content was reduced to <5% of control levels by incubation of 1321N1 cells with antimycin A at 37° for 20 min, then transferrin bound subsequently at 4° was not internalized when the temperature was raised to 37°. Podbilewicz and Mellman apparently could not carry out similar experiments with MDCK cells because even a 15-min exposure to NaCN and 2-deoxyglucose at 37° reduced cell surface receptors on MDCK cells almost to zero. The 37° protocol used by us (Fig. 4) caused only a 50% reduction in surface receptors in 1321N1 cells. Thus, the possibility remains that endocytosis of transferrin in MDCK cells would be blocked by reduction of the ATP content to sufficiently low levels (<5–10% of normal).

The observations that antimycin A prevents internalization of surface-bound transferrin and that the inhibition is immediately reversed by glucose, even in the continued presence of antimycin A, provide strong evidence that ATP is required for endocytosis *per se*. The different sensitivities of the internalization and externalization arms of the cycle provide a potential explanation for the disparate outcomes of previous studies addressing this question.

We were suprised to observe the rise in cell surface-bound [^{125}I]iodotransferrin that occurred when the antimycin A effect was reversed with glucose (Fig. 6). A possible explanation is that in ATP-depleted cells the pH in endosomes does not fall to a level sufficient to favor rapid dissociation of Fe^{3+} (20). Ciechanover *et al.* (21) showed that the rate of dissociation of apotransferrin from its receptor at neutral pH is about 30 times faster than the rate of dissociation of diferric transferrin. They concluded that, of any diferric transferrin recycled to the surface still bound to its receptor, only 30% would be expected to dissociate into the medium before being endocytosed into the cell. In their experiments NH_4Cl was used to increase the pH of endocytic vesicles, thus reducing the rate of dissociation of iron from transferrin (21). We speculate that in our experiments reduction in ATP content also increased endocytic vesicle pH (20) and allowed diferric transferrin bound to its receptor to be recycled to the cell surface. Because the rate of recycling was greater than the rate of dissociation of diferric transferrin, [^{125}I]iodotransferrin accumulated transiently at the cell surface.

For the past few years, we have attempted to determine the mechanism of catecholamine-induced translocation of β-adrenergic receptors (see Refs. 28 and 29). Our initial approach was to compare the effect of various agents and conditions on endocytosis of EGF and β-adrenergic receptors in the same cell line (7–9). However, one can question the validity of the EGF/receptor system as a model for β-adrenergic receptor internalization on at least two grounds. First, it is possible that EGF induces receptor polymerization before internalization (14–16), and the sensitivity of this reaction to test agents could confound the analysis of the requirements for endocytosis *per se*. Second, EGF and EGF receptors do not efficiently recycle, as do β-adrenergic receptors (32).

Thus, in this study we used internalization of [^{125}I]iodotransferrin as a model for receptor endocytosis. The evidence is unambiguous that transferrin is internalized via clathrin-coated pits (19–21). In addition, transferrin receptors are known to cycle continuously (17–18) and thus, unlike EGF receptors, provide a model for both internalization and externalization of ligand-bound β-adrenergic receptors (31).

Comparison of the effect of antimycin A on [^{125}I]iodotransferrin and β-adrenergic receptor diacytosis indicated no basis for distinction in the basal kinetics of endocytosis and exocytosis or in the effects of antimycin A on each process. The greater sensitivity of the exocytotic step to falling ATP content for each receptor type lends strong support to the idea that the two receptor types undergo similar translocation processes.

We have accumulated other evidence over the past few years strongly supporting the idea that β-adrenergic receptors undergo endocytosis via clathrin-coated pits. Thus, the sedimentation properties of vesicular structures containing internalized β-adrenergic receptors are similar to those of structures containing internalized [^{125}I]iodotransferrin (3) and [^{125}I]iodo-EGF (7), the limited accessibility of isolated internalized β-adrenergic receptors to hydrophilic ligands is most parsimoniously explained if the ligand binding site is in the lumen of endocytic vesicles (3, 33), agents known to inhibit clathrin-coated pit formation or stability (34–36) block transferrin receptor and β-adrenergic receptor diacytosis with similar characteristics (37), the kinetics of endocytosis ($t_{1/4}$, approximately 2 min) and exocytosis ($t_{1/4}$, approximately 4 min) of β-adrenergic receptors (2, 31) are similar to those of [^{125}]Iodotransferrin, and the effects of temperature also are similar.[3]

Although there is no consensus of results from previous morphological studies of ligand-induced β-adrenergic receptor redistribution in cells (38–42), recent studies by Von Zastrow and Kobilka (30) provide strong evidence that β-adrenergic receptors and transferrin receptors appear in the same population of intracellular sites after treatment of cells with a β-adrenergic receptor ligand.

The similarity in the patterns of effects of falling ATP content in 1321N1 cells on diacytosis of both transferrin and β-adrenergic receptors provides additional biochemical evidence for the similarity of the processes involved in metabolism of these two structurally distinct plasma membrane proteins.

[3] Liao and Perkins, Unpublished observations.

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