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Isoproterenol-Initiated β-Adrenergic Receptor Diacytosis in Cultured Cells

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SUMMARY

The kinetics of the return of internalized β-adrenergic receptors to the plasma membrane were measured in human astrocytoma cells. The movement of [^{125}I]iodopindolol-labeled receptors back to the plasma membrane was measured directly and was shown to occur with a $t_{1/2}$ of 3–4 min. Unlabeled receptors appeared to exhibit the same kinetics of externalization. The process was not inhibited by low concentrations (1–10 μM) of propranolol or even high concentrations of isoproterenol (0.1–1.0 mM). Higher concentrations of propranolol (0.1–1.0 mM) and other lipophilic amines inhibited externalization. The results are consistent with the proposal that catecholamine-induced β-adrenergic receptor internalization and externalization (diacytosis) occur via the clathrin-coated pit/endosome pathway.

When cells that express βAR are exposed to micromolar concentration of catecholamines, the receptors redistribute within the cell (1–4). In untreated cells, 85–95% of βAR detected by ligand binding are in the plasma membrane. In treated cells, however, only 15–50% of receptors are associated with the plasma membrane (2, 5, 6). The remainder are found to be associated with membranes that can be separated from plasma membranes by sucrose density gradient or differential centrifugation (1–3, 7). This agonist-induced translocation of βAR appears to be completely reversible upon removal of the catecholamine, because the associated ligand-induced changes in βAR-linked adenylyl cyclase activity are completely reversible and because receptors return to the original distribution (8, 9). It is our current working hypothesis that such βAR movements are the result of endocytosis via clathrin-coated pits and, like other receptors internalized by this pathway (e.g., transferrin receptor), internalized βAR can cycle back to the plasma membrane. In this report, we demonstrate directly the movement of internalized βAR back to the plasma membrane and provide an estimate of the $t_{1/2}$ for this process in human astrocytoma cells. The results provide further support for the working model of βAR translocation shown in Fig. 1.

Experimental Procedures

Materials. The following compounds were obtained from Sigma (St. Louis MO): (−)-isoproterenol (+)-bitartrate, 1-napthyl-*N*-ethylenediamine, Tris buffer, and DL-propranolol. Concanavalin A was from Calbiochem, ultrapure sucrose from Schwarz-Mann, and [^{125}I]NaI from Amersham. (−)-Pindolol was a gift from Gunther Engel of Sandoz Pharmaceuticals (Basel, Switzerland). Other chemicals used were of the highest grade available from commercial sources.

Cell growth. 1321N1 human astrocytoma cells were seeded from 7–10-day-old confluent cultures, in 10 ml of growth medium, into 100-mm plastic dishes at 10,000/cm^2. The cells were fed on the third day and used on the fourth day. The growth medium was Dulbecco's modified Eagle's medium containing 1.0 g/liter glucose, supplemented with 5% fetal bovine serum (GIBCO) and buffered with 3.7 g/liter $NaHCO_3$ and 8% CO_2.

Labeling of receptors in intact cells. Specific procedures are described in the figure legends. The general procedure is diagrammed in Fig. 1. The procedure utilized a high affinity, highly selective, lipophilic ^{125}I-labeled receptor antagonist, [^{125}I]iodopindolol, which binds to βAR irrespective of their location within the cell (10–12). The labeling time was kept short in order to reduce interference with the receptor cycling process *per se*. The short exposure time resulted in binding to about 15% of total receptors. Semiselective labeling of internalized βAR was achieved in the presence of hydophilic competitors, such as isoproterenol, which have access only to βAR on the cell surface (10–12).

Receptor binding assays. (−)-Pindolol was iodinated and [^{125}I] iodopindolol was purified as previously described (13). Binding assays consisted of 175-μl samples in binding buffer (145 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.4 at 37°), 50 μl of [^{125}I]iodopindolol at 40–100 pM in binding buffer, and 25 μl of drug in water or 1 mM HCl. Propranolol (1 μM) was used to define nonspecific binding, which averaged 4.5% of total binding. Incubations were for 30–60 min at 37°, and the binding reaction was terminated by addition of 8 ml of room temperature wash buffer (150 mM NaCl buffered with 10 mM Tris at

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ABBREVIATIONS: βAR, β-adrenergic receptor(s); EMEM, Eagle's minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate.

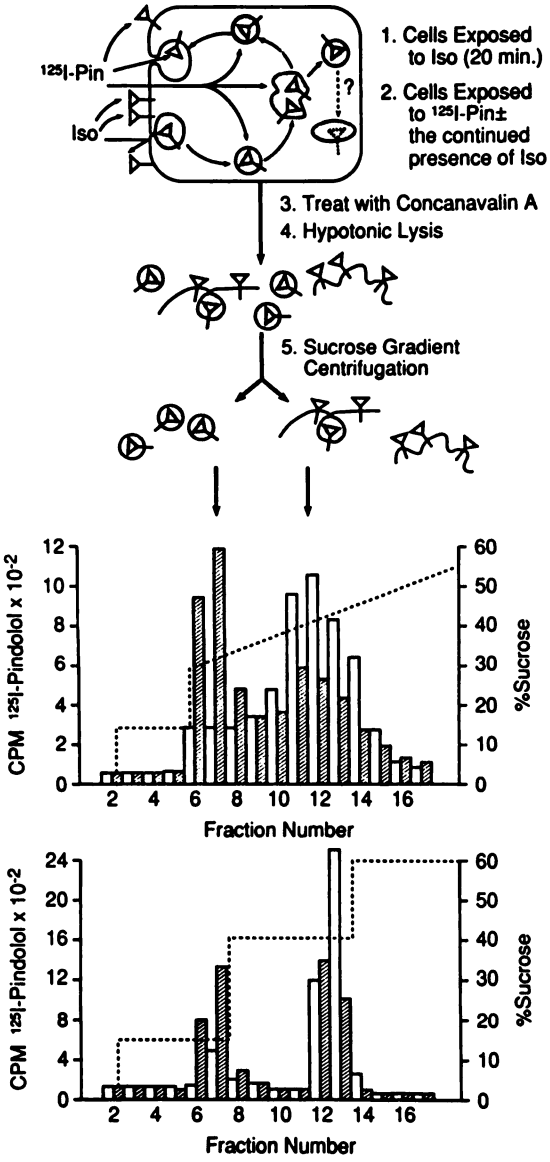

Fig. 1. Protocol for labeling of βAR in intact cells and separation of low density and high density receptor-containing membranes by sucrose density gradient centrifugation. The "cell diagram" indicates that [^{125}I] iodopindolol (^{125}*I-Pin*) can bind to all forms of βAR but that hydrophilic agents such as isoproterenol (*Iso*) compete for binding only at receptors on the cell surface. Thus, exposure of cells to [^{125}I]iodopindolol in the presence of isoproterenol would result in preferential labeling of βAR in the *shaded* subcellular structures. Hypotonic lysis and resolution of membrane species by sucrose density gradient centrifugation result in different patterns of distribution of βAR from cells treated or not with isoproterenol. The distribution shown in the two gradient profiles is that of total cellular βAR. Cells were treated or not with isoproterenol (0.1 μM) for 20 min, washed free of isoproterenol, and then exposed to 81 pM [^{125}I]iodopindolol for 30 sec at 37°. The labeled cells were then washed for 30 sec at 37° with HEPES/EMEM including 500 μg/ml concanavalin A and 1.0 μM propranolol. The cells were lysed in 4° hypotonic medium (1.0 mM Tris, 2.0 mM EDTA, pH 7.4), and the lysate was centrifuged over either step (*lower*) or continuous (*upper*) gradients of sucrose. Fractions (0.6 ml) were collected from the top of the gradients with an ISCO model 568 fractionater. The fractions were filtered over glass fiber filters and counted in a Tracor γ-counter. βAR distribution before (□) and after (▨) treatment of cells with isoproterenol. See legend to Fig. 2 for a more complete description of the preparation of labeled cells.

pH 7.8). The diluted sample was then filtered through a glass fiber filter, washed twice more, and counted dry in glass tubes in a Tracor γ-counter.

Sucrose gradients. Separation of receptors migrating with heavy or light density membranes was accomplished with either continuous or step gradients of sucrose buffered with 20 mM Tris at pH 7.4 at 2°. Step gradients were performed using a Beckman SW40 Ti rotor and polyallomer tubes; 4 ml of 60% sucrose were pipetted into each tube, followed by 4 ml of 38% sucrose and 4 ml of 15% sucrose. The entire cell lysate (up to 1 ml) was layered on top of the 15% sucrose. After centrifugation in a Beckman L8-70 or L5-65 ultracentrifuge for 1 hr at 2° and 35,000 rpm (112,000 × *g*), the gradient was fractionated into cold 13- × 100-mm glass tubes, using an apparatus that aspirated sequentially to 2.0 cm (discarded supernatant), to 4.9 cm (light peak), and to 7.5 cm (heavy peak). The system was designed to collect the visible upper (light peak) and lower (heavy peak) bands, by collecting about 2 ml of sucrose on both sides of each band. To assure measurement of only membrane-bound radioactivity, the fractions were filtered over 25-mm glass fiber filters, the tube was rinsed once, the filter was washed twice with room temperature wash buffer, and the filters were counted. The supernatant and nuclear pellet (that left in the centrifuge tube) combined contained <4% of the total filterable radioactivity. For continuous gradients, 9 ml of a 30–60% gradient of sucrose were prepared in the centrifuge tube, using an ISCO gradient former. Two milliliters of 15% sucrose were layered over the gradient, and the sample (lysate) was layered on top of the 15% sucrose. Centrifugation was as for the step gradients.

Previous work has shown that treatment of 1321N1 cells with concanavalin A before lysis results in the formation of large sheets of plasma membrane (14). Concanavalin A apparently stabilizes the plasma membrane to fragmentation and inhibits vesiculation upon cell lysis (15). We presume that βAR in the light density fractions are in vesicles containing cytoplasm; however, very small, open membrane fragments also would not sediment into the denser portions of the gradient. In the experiments reported here, cells were treated with concanavalin A before lysis in order to optimize the resolution of the two subpopulations of βAR.

Fig. 1 compares the receptor distribution of lysates of naive cells with that of cells treated for 20 min with isoproterenol on continuous and step gradients, when these gradients were fractionated into 0.6-ml fractions. The shift of receptors from heavy to light density membranes is readily apparent on either gradient; the large separation between heavy and light density membranes on the step gradients allowed the simple fractionation technique described above to be reproducible and effective.

Results

In earlier studies, we used sucrose density gradient centrifugation to establish the kinetics of agonist-induced internalization of βAR (2). Such receptors migrate to lower densities of sucrose (less than 35% or 1.13 g/ml) than do the bulk of receptors in untreated cells (45% sucrose or 1.17 g/ml). Treatment of 1321N1 cells with 1 μM isoproterenol caused appearance of βAR in the light density fractions, after a lag of about 60 sec, with a $t_{1/2}$ of about 90–120 sec (2). In 1321N1 cells, βAR achieved a steady state distribution after 10 min of exposure to isoproterenol, of about 50:50 in light and heavy density fractions, with little or no loss of total binding sites. Because the overall reaction is completely reversible, the steady state redistribution could be explained if βAR continually cycle between plasma membrane and intracellular membranes.

Evidence for reversal of agonist-induced βAR redistribution is shown in Fig. 2. In this experiment, cells were exposed to isoproterenol for 20 min and then washed free of catecholamine, and the incubation was continued at 37°. At the times indicated,

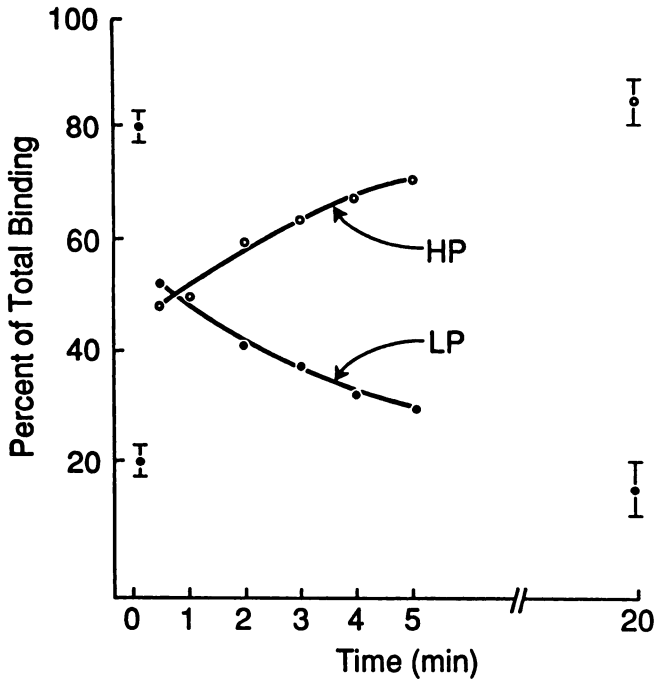

Fig. 2. Change in receptor distribution with time after wash-out of isoproterenol. Cells in 100-mm dishes were incubated at 37° with 1 mM ascorbate in HEPES-buffered (20 mM) EMEM, in the presence or absence of 0.1 μM isoproterenol, for 20 min. Dishes were then washed two times with 8 ml of HEPES/EMEM at 37°. Each wash required 10 sec. Incubation was continued for the times indicated in the figure. Receptors were labeled by exposure of the cells to 74 pM [^{125}I]iodopindolol in HEPES/EMEM for exactly 30 sec, beginning at the times indicated. The labeling medium was aspirated at 25 sec and wash buffer (HEPES/EMEM plus 1 μM propranolol, 37°) was added to end the labeling period at 30 sec. The cells were treated with 1 μM propranolol and 500 μg/ml concanavalin A in HEPES/EMEM at 37° for 30 sec and then washed once with 10 ml of 4° hypotonic buffer (1 mM Tris plus 2 mM EDTA, pH 7.4). After a 20-min incubation in this buffer at 4°, the medium was aspirated from the swollen cells and they were scraped from the surface with a rubber spatula. This sample and a 0.5-ml wash of the scraped dish were combined to generate about 0.9 ml of lysate that was layered on the sucrose step gradient described in Experimental Procedures. The values plotted (0.5–5.0 min) are the averages of duplicate determinations from a representative experiment. The values at 0 and 20 min are the mean ± standard deviation of four similar but independent experiments. Values at 0 time were obtained by exposing naive cells to the labeling procedure described above. *LP* and *HP*, upper (*LP*) and lower (*HP*) visible bands on the step gradients, as described in Experimental Procedures.

the cells were exposed to [^{125}I]iodopindolol for 30 sec, washed, and lysed, and the lysate was centrifuged over a gradient of sucrose. Receptor content in the light fractions declined from 52% to 15% over the same time course as the receptor content of the heavy fraction increased from 48% to 85%. No significant change in total number of receptors occurred during the experiment. The $t_{1/2}$ for the redistribution of βAR was about 4 min. Although the gain in βAR in the heavy fractions appears to be at the expense of loss from the light fractions, such experiments do not prove that direct translocation occurred, nor can one conclude that receptor cycling was occurring during the 20-min exposure to isoproterenol.

Proof of βAR cycling is provided by observing the fate of radioligand-bound receptors after labeling in intact cells. We had shown previously (12) that brief exposure of agonist-treated 1321N1 cells to [^{125}I]iodopindolol at 37° (in the continuing presence of isoproterenol) resulted in preferential labeling of the βAR that subsequently were found in the lower density fractions. This result was interpreted to indicate that hydrophilic compounds such as isoproterenol readily compete with the binding of lipophilic radioligands at the cell surface (heavy peak) but do not compete well at internalized receptors (light peak). The concept is illustrated in Fig. 1. Such a labeling protocol allowed us to semiselectively label internalized receptors and follow their fate. The addition of 1 μM propranolol after the 1-min exposure to [^{125}I]iodopindolol not only stopped the binding reaction at all sites (propranolol is lipophilic) but also prevented rebinding of any label that dissociated from βAR. Based on studies using cell lysates, the $t_{1/2}$ for dissociation of iodopindolol at 37° is about 10 min; thus, the majority of labeled receptors should remain labeled throughout the first 6 min of the experiment. The appearance of label in the heavy peak fractions and loss from the light peak is shown in Fig. 3A as a percentage of the total of radioactivity in the two fractions. The data in Fig. 3A are from a single experiment, whereas in Fig. 3B we show mean values from six similar experiments in which change in βAR distribution was measured 4 and 12 min after labeling. It is clear that, not only did βAR increase in the

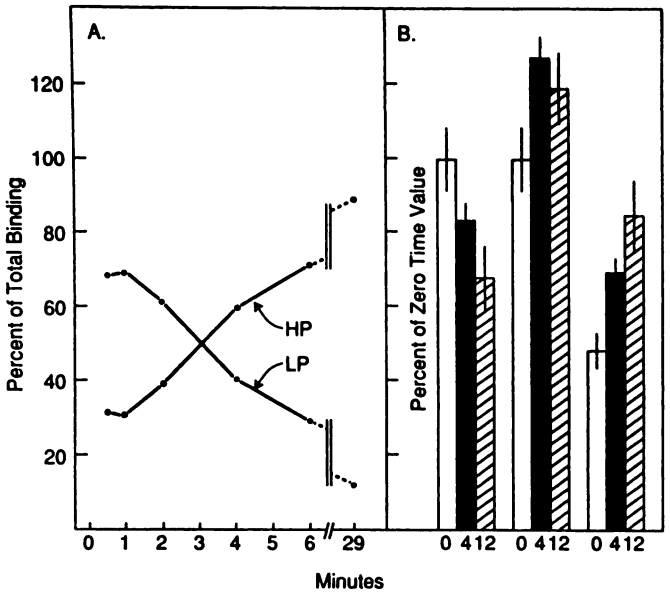

Fig. 3. A, Time course of externalization of [^{125}I]iodopindolol-labeled βAR. After a 20-min exposure to 1.0 μM isoproterenol, cells were labeled by adding [^{125}I]iodopindolol (94 pM) to the medium in the continued presence of isoproterenol. At the end of the 60-sec labeling period, propranolol (1.0 μM) was added and the incubation was continued for up to 29 min. Replicate cultures received propranolol at the beginning of the labeling period, in order to assess nonspecific binding. At the times indicated, the dishes were washed three times with HEPES/EMEM containing 100 μM propranolol and were then exposed to concanavalin A (250 μg/ml) at 4° for 5 min. Lysis of cells and centrifugation of the lysate were as described for continuous gradients in Experimental Procedures and in the legend to Fig. 1. Pooled fractions 5–8 and 9–15 were designated light peak (*LP*) and heavy peak (*HP*), respectively. Nonspecific binding (<5%) was subtracted. Fractions were not filtered before counting. B, In six similar experiments, the change in the total amount of bound [^{125}I]iodopindolol was measured in light peak and heavy peak fractions, as described for step gradients of sucrose. *Left*, total bound radioligand at 0, 4, and 12 min, expressed as percentage of the 0 time value. *Center*, total bound radioligand in the heavy peak at 0, 4, and 12 min expressed as percentage of the 0 time value. *Right*, bound radioligand in the heavy peak as percentage of the total remaining bound radioligand. Values are the average of eight determinations from six individual experiments. *Vertical bars*, standard deviation.

heavy peak as a percentage of total label remaining, but the absolute amount of bound ligand in the heavy peak also increased. These results were consistent with a dissociation $t_{1/2}$ for [^{125}I]iodopindolol of 21 min from whole cells, a value about 2 times that derived from the cell lysate measurements mentioned above.

These results provide direct, unambiguous evidence that antagonist-bound βAR undergo recycling to the plasma membrane. The $t_{1/2}$ for ligand-βAR externalization (Fig. 3) is the same (3–4 min) as the $t_{1/2}$ for reappearance of βAR in the plasma membrane upon removal of agonist (Fig. 2). We conclude that there is a similar mode of externalization for βAR whether or not the receptor is bound to antagonist.

As presented, both methods of observing βAR externalization exclude the effective presence of isoproterenol. The type of experiment illustrated in Fig. 2 required removal of isoproterenol to prevent internalization of the recycling βAR. The prelabeling experiments (Fig. 3) utilized 1 μM propranolol to terminate the pulse of labeling by [^{125}I]iodopindolol, but it also prevented any subsequent effect of isoproterenol. We thus performed a series of experiments attempting to terminate the [^{125}I]iodopindolol labeling rapidly by washing, followed by incubation with or without isoproterenol, and measured βAR movement to the plasma membrane.

In Fig. 4 we show that isoproterenol (0.1 μM) apparently has no effect on the rate of externalization of [^{125}I]iodopindolol-labeled βAR. In similar experiments, 1.0 and 10 μM isoproterenol gave similar results (data not shown). However, simple interpretation of the data is compromised by the observation that total bound radioligand (light peak plus heavy peak) did not decrease significantly during the time course of the experiment. As indicated previously, the apparent dissociation of radioligand in the presence of propranolol (Fig. 3) occurred with a $t_{1/2}$ of 21 min; thus, by 12 min a decline of about 38% was expected for the experiment in Fig. 4. Also, in the presence of 0.1–10 μM isoproterenol the extent of reduction in βAR-bound radioligand by 12 min was less (16–18% of total) than in the presence of 1.0 μM propranolol (10–14% of total). Such results suggest to us that the washing procedures used in the type of experiment shown in Fig. 4 did not eliminate all free [^{125}I]iodopindolol and binding to intracellular βAR continued to occur. Binding to βAR on the cell surface would have been effectively blocked by 0.1–10 μM isoproterenol. Thus, the $t_{1/2}$ for redistribution is not necessarily comparable with values generated by the experiments illustrated in Figs. 2 and 3. Nonetheless, the observed $t_{1/2}$ values are similar for the three types of experiments.

When experiments similar to that of Fig. 4 were carried out in the presence of 0.1 and 1.0 mM isoproterenol during the βAR redistribution stage, total radioligand bound was reduced to the same level as in the presence of 1.0 μM propranolol, and the $t_{1/2}$ for externalization also was the same as in the presence of propranolol (data not shown). The results of these two sets of experiments lead us to the conclusion that isoproterenol does not affect the rate of externalization of antagonist-bound βAR.

The effect of a number of substances on βAR externalization was tested. The protocol used involved termination of [^{125}I] iodopindolol pulse-labeling of desensitized cells with propranolol. Thus, compounds to be tested for effects on externalization were added in addition to 1 μM propranolol, which did not appear to affect this process. Data are expressed as the percentage of total label found in the light peak after sucrose gradient fractionation of a lysate from a 12-min incubation at 37° (Table 1). The results indicate that lipophilic amines in general inhibited the externalization of βAR.

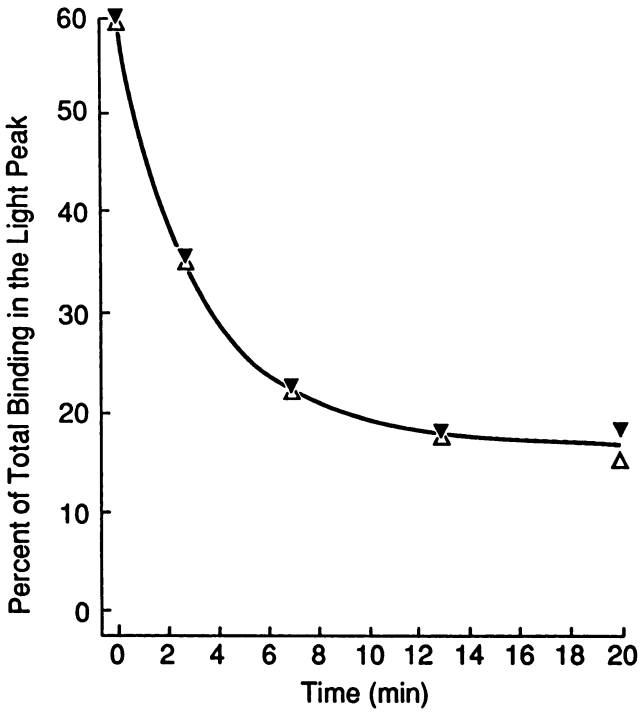

Fig. 4. Effect of isoproterenol on externalization of [^{125}I]iodopindolol-labeled βAR. Cells were incubated with 0.1 μM isoproterenol and 1.0 mM ascorbate in HEPES/EMEM for 20 min at 37°. Labeling was effected by addition of [^{125}I]iodopindolol (105 pM) and additional isoproterenol to bring the final concentration to 3.0 μM. Labeling was for 30 sec. The cells were then washed three times with HEPES/EMEM and incubated in a fourth volume of the same medium, with (▼) or without (△) 0.1 μM isoproterenol, for up to 20 min at 37°. The cells were lysed and the lysate was centrifuged over a step gradient of sucrose, as described in Experimental Procedures and in the legend to Fig. 2. The time indicated is from the end of the 30-sec exposure to the radioligand. The result of this experiment was replicated in a total of three experiments at three concentrations of isoproterenol in the recovery medium (0.1, 1.0, and 10 μM isoproterenol).

The effect of propranolol on externalization was examined in more detail. As shown in Fig. 5, inhibition does not occur at concentrations below 10 μM, but externalization is essentially completely blocked by 1.0 mM DL-propranolol.

In view of the fact that the luminal pH of endosomes is low and we are proposing that βAR in such structures are labeled with [^{125}I]iodopindolol, it was important to determine the effect of pH on the binding of this radioligand. It is evident from the results shown in Fig. 6 that, between pH 5.5 and 6.5 (the range of pH in endosomes), ligand binding is reduced 75–30%.

Discussion

The results presented here provide the first direct evidence that so-called sequestered (6, 16) or internalized (4, 11, 12) βAR are capable of returning to the plasma membrance with or without bound ligand and in the presence or absence of isoproterenol. The kinetics of this putative externalization reaction for βAR are similar to the kinetics of exocytosis for other receptors that undergo diacytosis via the clathrin-coated pit/endosone pathway (17, 18). To date, our numerous attempts to

TABLE 1

Effect of various amines on βAR externalization

The data in this table are derived from three experiments. All experiments used cells after exposure to 0.1 μM isoproterenol for 20 min. The cells were washed twice and exposed to [^{125}I]iodopindolol for 30 sec in the absence of competitor (experiments B and C) or in the presence of 0.1 μM CGP-12177 (experiment A), a hydrophilic antagonist that, like isoproterenol, allows preferential labeling of the light peak fraction of βAR. Cells were treated further as described in the legend to Fig. 2. The light peak contained 50% of total βAR at zero time in experiment A, 44% in experiment B, and 49% in experiment C. Externalization was allowed to occur at 37° for 12 min in the presence of 1 μM propranolol plus the various additives. Values represent the percentage of labeled βAR present in the light peak fraction after 12 min. Values are averages of close duplicates in experiments A and B and single measurements in experiment C. Twenty-four-minute measurements (not shown) in experiment C confirmed those made at 12 min. The effects of D- and L-propranolol in experiment B were confirmed in experiment C (not shown).

Addition	Light peak	Inhibition
	%	%
Expt. A		
None	14	0
Methylamine (30 mM)	20	19
Methylamine (100 mM)	30	44
DL-Propranolol (0.1 mM)	44	83
Expt. B		
None	13	0
D-Propranolol (0.1 mM)	34	68
L-Propranolol (0.1 mM)	29	52
Expt. C		
None	19	0
Methylamine (30 mM)	24	17
Phentolamine (0.1 mM)	25	20
N-1-Napthylethylenediamine (0.1 mM)	37	60

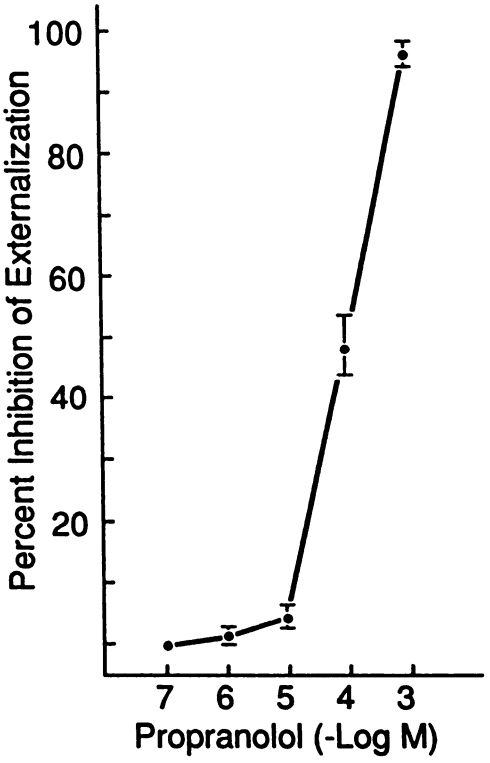

Fig. 5. Effect of DL-propranolol on externalization of βAR. Cells were exposed to isoproterenol (0.1 μM) for 20 min, washed, and labeled with [^{125}I]iodopindolol for 30 sec. Labeling was terminated by washing and addition of the indicated concentration of DL-propranolol in HEPES/EMEM. After 12 min, the cells were prepared for lysis and the lysate was centrifuged over a step gradient of sucrose, as described in Experimental Procedures and in the legend to Fig. 2. *Verticle bars*, range of duplicate determinations. Similar results are reported in Table 1.

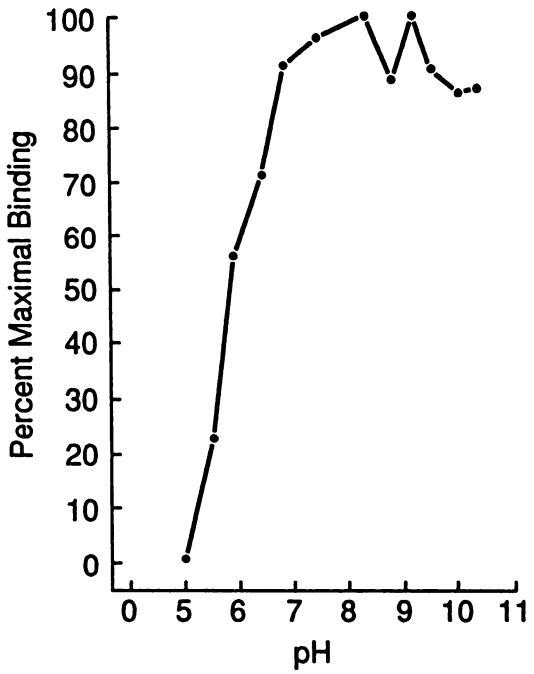

Fig. 6. Effect of pH on [^{125}I]iodopindolol binding. Cells were exposed to 1.0 mM ascorbate in HEPES/EMEM. Lysates were prepared as described, then frozen, thawed, and diluted 1/4 with binding buffer at 12 different pH values. The final pH after dilution with lysate was determined and varied from 5.03 to 10.38. The pH of the binding buffer was varied by addition of acetic acid or 10 M NaOH. Assays were done in triplicate at each pH. Binding in the presence of 1.0 μM propranolol was <3% of maximal binding and did not vary when measured at pH 5.03, 7.48, 8.42, and 10.38. Binding was for 45 min at 37° with 46 pM [^{125}I]iodopindolol. Binding buffer was brought to pH 4.63 or 10.38 and then back to pH 8.5, in order to test the effect of the added salts; binding in such solutions was 90% of maximal. This experiment was carried out three times with similar results. The results were not influenced by the buffer used, for a given range of pH values.

distinguish the pathway of agonist-induced βAR translocation from that mediating endocytosis of epidermal growth factor or diacytosis of transferrin have only led to further evidence of similarity. Thus, the kinetics of internalization are similar ($t_{1/2}$ = 2 min, after a lag of 45–60 sec) (2), the kinetics of exocytosis and the effect of lipophilic amines on exocytosis are similar (this work), the inhibitory effects of phenylarsine oxide (19, 20), antimycin A (21), reduced temperature,[2] hypertonicity (22), reduced intracellular pH (22), and reduced cellular K^+ (22) are similar, and the apparent requirement of βAR internalization for loss to occur (22) and the hydrodynamic properties of the vesicles containing internalized βAR (1, 2), as well as their permeability properties (11, 12), are all consistent with the involvement of the clathrin-coated pit/endosome pathway in agonist-induced βAR translocation.

Although our results establish the capacity of internalized βAR to cycle to the cell surface, we have not unambiguously shown that receptor cycling occurs continuously during exposure to isoproterenol. An alternate possibility might be that catecholamines act by inhibiting the rate of exocytosis of constitutively cycling βAR. Thus, upon addition of an agonist, the cytosolic content of βAR would rise, due to selective inhibition

[2] G. L. Waldo, Y. Fujioka, L. Kieffer, Y. F. Liao, T. K. Harden, and J. P. Perkins. The inhibitory effect of lipophiliic amines and reduced temperature on down-regulation of β-adrenergic receptors. Submitted for publication.

of one half of a cyclic reaction. Of course, our results demand that radioligand-bound βAR are exempt from such inhibition of exocytosis. Also, our unpublished data argue against this possibility, because circumstances that selectively inhibit the exocytotic arm of transferrin receptor cycling (reduced temperature or reduced ATP content) do not cause the expected rise in cytosolic βAR in control cells. However, such circumstances do cause a further rise in cytosolic βAR when applied to isoproterenol-treated cells.[3] Thus, the most parsimonious interpretation of results to date is that βAR exhibit little or no constitutive diacytosis but are induced to enter into clathrin-coated pits by catecholamines and undergo diacytosis continually in the presence of such receptor agonists.

Because the internal pH of the endosome is known to range from 6.5 to 5.0 and to exhibit a gradient favoring lower pH in "late" versus "early" endosomes (23, 24), it is of interest to consider the binding properties of βAR radioligands in this range of pH. Assuming that efficiently recycling receptors will be restricted to early endosomes (24), we can presume that the pH in the environment of internalized βAR will be at the upper extreme of the stated pH range. This argument and the results shown in Fig. 6 indicate that the binding of [^{125}I]iodopindolol would be reduced 30–40% in such early endosomes. However, because it is a lipophilic amine, [^{125}I]iodopindolol would be concentrated in acidic environments. Although we cannot accurately estimate the outcome of these opposing effects on binding of the radioligand to internalized βAR, J. F. Liao in our laboratory has shown that the apparent K_D for [^{125}I]iodocyanopindolol binding to βAR in the light peak fractions prepared from desensitized ROS 17/2.8 cells is increased about 2-fold, relative to the K_D for binding to βAR in the heavy peak fractions (22).

Propranolol at relatively low concentrations (1.0–10 μM) appeared not to affect the rate of externalization of βAR; however, at higher concentrations (0.1–1.0 mM) it caused essentially complete inhibition (Fig. 5). D- and L-propranolol were equally effective as inhibitors of βAR externalization. These observations suggest to us that the inhibitory action of propranolol is not mediated by receptor binding but may reflect the capacity of lipophilic amines to influence vesicular pH (23, 25). Other lipophilic amines also inhibited the externalization of βAR and have been shown to have selective effects on the exocytic arm of diacytosis of transferrin (17). Alternatively, the well known membrane-disruptive effects of propranolol at millimolar concentrations could block externalization nonspecifically.

Taken together, our results indicate that internalized βAR exist in an environment that favors access to lipophilic ligands but reduces the apparent affinity for antagonist radioligands. Of course, it is not clear that it is the pH of the environment of light peak βAR that reduces affinity for the radioligand. Such a change in binding could reflect covalent modification of the receptor *per se*, e.g., by phosphorylation (26, 27).

The βAR exhibits properties that distinguish it from other types of receptors that undergo endocytosis. Some receptors, such as those for transferrin and asialoglycoprotein, appear to cycle continuously even in the absence of a bound ligand (18). Other receptors, such as those for epidermal growth factor and insulin, require bound ligand to initiate internalization (18). Receptors in the latter category are minimally recycled, and endocytosis results in rapid degradation of the receptor protein (18). The βAR exhibits hybrid features, in that it appears not to undergo significant internalization in the absence of its ligand but in the presence of a catecholamine it undergoes diacytosis continuously for up to 1 hr without detectable down-regulation of receptor content. Whether this behavior indicates a unique property of the βAR *per se* or reflects its utilization of a similar but different pathway for diacytosis than that used by other receptors awaits unambiguous determination of the subcellular structures associated with internalization of the βAR.

[3] J. F. Liao and J. P. Perkins, unpublished observations.

References

1. Harden, T. K., C. U. Cotton, G. L. Waldo, J. K. Lutton, and J. P. Perkins. Catecholamine-induced alteration in the sedimentation behavior of membrane-bound β-adrenergic receptors. *Science (Washington D. C.)* **210**:441–443 (1980).
2. Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**:13900–13908 (1983).
3. Stadel, J. M., B. Strulovici, P. Nambi, T. N. Lavin, M. M. Briggs, M. G. Caron, and R. J. Lefkowitz. Desensitization of the β-adrenergic receptor of frog erythrocytes: recovery and characterization of the down-regulated receptors in sequestered vesicles. *J. Biol. Chem.* **258**:3032–3038 (1983).
4. Hertel, C., and J. P. Perkins. Receptor-specific mechanisms of desensitization of β-adrenergic receptor function. *Mol. Cell Endocrinol.* **37**:245–256 (1984).
5. Strader, C. D., I. S. Sigal, A. D. Blake, A. H. Cheung, R. B. Register, E. Rands, B. A. Zemcik, M. R. Candelore, and R. A. F. Dixon. The carboxyl terminus of the hamster β-adrenergic receptor in mouse L cells is not required for receptor sequestration. *Cell* **49**:855–863 (1987).
6. Bouvier, M., W. P. Hausdorff, A. De Blasi, B. F. O'Dowd, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. Removal of phosphorylation sites from the β-2 adrenergic receptor delays onset of agonist-promoted desensitization. *Nature (Lond.)* **333**:370–373 (1988).
7. Kassis, S., and M. Sullivan. Desensitization of the mammalian β-adrenergic receptor: analysis of receptor redistribution on non-linear sucrose gradients. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:35–46 (1986).
8. Su, Y.-F., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. *J. Biol. Chem.* **255**:7410–7419 (1980).
9. Frederich, R. C., G. L. Waldo, T. K. Harden, and J. P. Perkins. Characterization of agonist-induced β-adrenergic receptor specific desensitization in C62B glioma cells. *J. Cyclic Nucleotide Res.* **9**:103–118 (1983).
10. Staehelin, M., and P. Simons. Rapid and reversible disappearance of β-adrenergic cell surface receptors. *EMBO J.* **1**:187–190 (1982).
11. Hertel, C., M. Staehelin, and J. P. Perkins. Evidence for intravesicular β-adrenergic receptors in membrane fractions from desensitized cells: binding of the hydrophilic ligand CGP-12177 only in the presence of alamethicin. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9**:119–128 (1983).
12. Toews, M. L., G. L. Waldo, T. K. Harden, and J. P. Perkins. Relation between agonist-induced low affinity β-adrenergic receptors and an agonist-induced, low density vesicular form of the receptor. *J. Biol. Chem.* **259**:11844–11850 (1984).
13. Barovsky, K., and G. Brooker. ^{125}I-Iodopindolol, a new highly selective radioiodinated *beta*-adrenergic receptor antagonist: measurement of *beta* receptors on intact rat astrocytoma cells. *J. Cyclic Nucleotide Res.* **6**:297–307 (1980).
14. Lutton, J. K., R. C. Frederich, and J. P. Perkins. Isolation of adenylate cyclase-enriched membranes from mammalian cells using concanavalin A. *J. Biol. Chem.* **254**:11181–11184 (1979).
15. Scarborough, G. A. Isolation and characterization of *Neurospora crassa* plasma membranes. *J. Biol. Chem.* **250**:1106–1111 (1975).
16. Sibley, D. R., and R. J. Lefkowitz. Molecular mechanism of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature (Lond.)* **317**:124–129 (1985).
17. Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line: effect of lysosomotropic agents. *J. Biol. Chem.* **258**:9681–9689 (1983).
18. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1**:1–39 (1985).
19. Hertel, C., S. J. Coulter, and J. P. Perkins. A comparison of catecholamine-induced internalization of β-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytana cells: inhibition by phenylarsine oxide. *J. Biol. Chem.* **260**:12547–12553 (1985).
20. Kassis, S., M. Olasmaa, M. Sullivan, and P. H. Fishman. Desensitization of the β-adrenergic receptor-coupled adenylate cyclase in cultured mammalian

cells: receptor sequestration versus receptor function. *J. Biol. Chem.* **261**:12233–12237 (1986).
21. Hertel, C., S. J. Coulter, and J. P. Perkins. The involvement of cellular ATP in receptor-mediated internalization of epidermal growth factor and hormone-induced internalization of β-adrenergic receptors. *J. Biol. Chem.* **261**:5974–5980 (1986).
22. Liao, J. F. Characterization of agonist-induced internalization of β-adrenergic receptors and its relation to down-regulation and desensitization. Ph.D. dissertation, Yale University (1990).
23. Dean, R. T., W. Jessup, and C. R. Roberts. Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.* **217**:27–40 (1984).
24. Mellman, I., R. Fuchs, and A. Helenius. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55**:663–700 (1986).
25. Dulis, B., and B. Wilson. The uptake of amines by polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **643**:398–406 (1981).
26. Clark, R. B., M. W. Kunkel, J. Friedman, T. J. Goka, and J. A. Johnson. Activation of cyclic AMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. USA* **85**:1442–1446 (1988).
27. Hausdorf, W. P., M. Bouvier, B. F. O'Dowd, G. P. Irons, M. G. Caron, and R. J. Lefkowitz. Phosphorylation sites on two domains of the β-2 adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* **264**:12657–12665 (1989).

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