A Comparison of Catecholamine-induced Internalization of 
\(\beta\)-Adrenergic Receptors and Receptor-mediated Endocytosis 
of Epidermal Growth Factor in Human Astrocytoma Cells 

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The ligand-induced internalization of \(\beta\)-adrenergic receptors and the receptor-mediated internalization of epidermal growth factor were blocked, under similar conditions, by phenylarsine oxide (PAO) in human astrocytoma cells (1321N1). The inhibition was not prevented or reversed by monofunctional sulfhydryl agents such as 2-mercaptoethanol or glutathione; however, the inhibitory action of PAO was blocked and reversed by bifunctional thiol such as 2,3-dimercaptopropanol or dithiothreitol. The results are consistent with the interaction of PAO with vicinal sulfhydryl groups to form a stable ring structure. PAO did not prevent isoproterenol-induced uncoupling (desensitization) of \(\beta\)-adrenergic receptors even though receptor internalization was completely blocked. The effects of PAO on receptor internalization could not be explained by any action of the trivalent arsenical to lower ATP levels. Ligand binding to both receptors was not detectably altered by PAO under conditions selective for inhibition for endocytosis. The results suggest a common mechanism for internalization of \(\beta\)-adrenergic receptors and epidermal growth factor by a process that involves vicinal sulfhydryl groups.

The activation by catecholamines of the \(\beta\)-adrenergic receptor (BAR)-linked adenylyl cyclase is a transient effect in most cells. Following rapidly after the activation process, a set of temporally related events occur that lead to loss of response of the cell to catecholamines. As a part of this desensitization process, a portion of the cell surface BAR was shown to change form in a manner consistent with receptor internalization (1-4). Thus, BAR can be isolated from desensitized cells in a membrane form depleted of other plasma membrane marker proteins. Such receptors are poorly accessible to hydrophilic ligands such as isoproterenol (3) and CGP-12177 (4); whereas, BAR on the surface of intact cells or in lysates from control cells are readily accessible to these hydrophilic ligands. These observations have been interpreted as consistent with the proposal that the catecholamine-induced form of BAR represents BAR with the ligand binding site on the inside of endocytotic vesicles. It follows that BAR might be internalized and processed in a manner similar to receptor-mediated endocytosis of certain polypeptides (asialoglycoprotein, insulin, EGF, low density lipoprotein, etc; for review, see Ref. 5). Internalization of protein-receptor complexes appears to involve sequestration in clathrin-coated pits and internalization by endocytosis in vesicles which simultaneously can contain different protein-receptor complexes (6, 7).

The processing of polypeptide ligand and receptor appears to vary. In some cases the receptor is efficiently shuttled back to the cell surface 1) upon dissociation of the ligand and its degradation such as for asialoglycoprotein (8) and low density lipoprotein (9), or 2) with the protein ligand intact such as for transferrin (10). In some cases both ligand and receptor are degraded (11). There is evidence that separation of a ligand destined for degradation (asialoglycoprotein) from the receptor destined to recycle to the cell surface occurs in tubular organelles termed "compartments of uncoupling of receptor and ligand" (8).

In this report we compare the effects of the trivalent arsenical, phenylarsine oxide (PAO) on catecholamine-induced internalization of BAR and receptor mediated internalization of EGF in the human astrocytoma cell line 1321N1. PAO previously had been shown to inhibit internalization of a variety of proteins (12-14) including EGF (15). We demonstrate that 1) PAO inhibits the internalization of both BAR and EGF, 2) the effect of PAO is reversed or prevented by bifunctional but not monofunctional sulfhydryl agents, and 3) the effect of PAO cannot be explained on the basis of inhibition of energy metabolism.

EXPERIMENTAL PROCEDURES

Materials—(-)-isoproterenol bitartrate, antymycin A, and 2-deoxyglucose were obtained from Sigma; epidermal growth factor from Biomedical Technologies, Cambridge, MA; ATP bioluminescence CLS kits from Boehringer Mannheim; phenylarsine oxide from Aldrich. Phenylarsine oxide was dissolved in dimethyl sulfoxide and diluted to a final concentration of the solvent of 0.1%. The following drugs were gifts: (-)-cyanopindolol from Sandoz, CGP-12177 from Dr. M. Stachelin, Basel. \(\text{[14}^\text{C}]\)-cyanopindolol (CYP) was prepared by a modification of the method of Barovsky and Brooker (16). \(\text{[125}^\text{I}]\)-EGF was prepared by the chloramine-T method (17). \(\text{[3}^\text{H}]\)ATP was synthesized as described (18).

Cell Culture—Human astrocytoma cells (1321N1) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Growth of cells was carried out under an atmosphere of
92% air and 8% CO₂ at 37 °C in a humidified incubator. Cells were seeded at 1.5 × 10⁵ cells/cm² and were subcultured 4 days prior to the experiment.

**Binding of Cyanopindolol**—To determine the total number of BAR, lysates or membranes were incubated with 50 pm (10 × 10⁶) [125I]-CYP in Hepes-NaCl, 20 mm Tris, 5 mm MgCl₂, pH 7.4, in a total volume of 0.25 ml for 2 h at 30 °C. Non-specific binding was determined in the presence of 1 μM propranolol.

**Internalization of BAR**—Cells were incubated with 1 μM (-)-isoproterenol for 20 min at 37 °C to induce receptor internalization. The internalized form of BAR was detected by two different methods: 1) competitive binding assay, in which desensitized cells were washed with Heps-Eagle's medium incubated with 0.25 mg/ml concanavalin A (19) for 20 min at 4 °C then lysed by hypotonic shock in 1 mM Tris, 2 mM EDTA, pH 7.4, for 20 min at 4 °C. 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 centrifuged for 30 min at 55,000 rpm in a Beckman SW 40 rotor. The interfaces were collected and diluted 1:1 with 154 mM NaCl, 20 mM Tris, 5 mM MgCl₂, pH 7.4, and BAR was determined by binding of [125I]-CYP. Internalized BAR migrated preferentially at the 15–38% interface and BAR on the plasma membrane at the 38–60% interface. 2) Competition binding, in which desensitized cells were washed and lysed by hypotonic shock in 1 mM Tris, 2 mM EDTA, pH 7.4, for 20 min at 4 °C. Competition binding was carried out with diluted lysates using 10 pm [125I]-CYP and a range of concentrations of CGP-121177 (incubation was carried out at 30 °C for 2 h (20)). Internalized BAR (vesicles in the lysate) exhibited a marked inaccessibility to CGP-121177. A rapid increase in [125I]-CYP binding in the presence of 50 nm CGP-121177 provided a quantitation of internalized BAR (see Fig. 5).

**Epidermal Growth Factor Binding**—Intact 1321N1 cells were washed once with Heps-Eagle's medium containing 0.1% bovine serum albumin and incubated with 4 ng/ml (10⁶ cpm/ml) EGF. The incubation was either performed at 37 °C for up to 120 min or, to determine a single cycle of internalization, cells were incubated with [125I]-EGF for 2 h at 4 °C, washed, and then incubated at 37 °C with nonlabeled EGF in the same buffer. After incubation at 37 °C, cells were chilled and washed five times with assay buffer. To determine total binding, cells were solubilized in 0.1 N NaOH, and cell-associated radioactivity was determined in a y-counter. To determine internalized EGF, cells were washed for 5 min with 20 mM sodium acetate, pH 4.5, to remove cell-surface-bound EGF, washed, and lysed afterwards thoroughly with assay buffer to readjust the pH 7.4 before lysis. Centrifugation was as described above for BAR.

**Determination of the Intracellular ATP Concentration**—Cells were solubilized by addition of 1.5 ml of 70 °C 0.14 N HNO₃ to each dish. Dishes were incubated for an additional 20 min at 37 °C to allow complete solubilization of the supernatant. The supernatant was collected and centrifuged for 10 min at 4000 × g. The pH of the supernatant was adjusted to 7.0 by adding 0.6 N NaOH in 20 mM Tris.

ATP content was determined using a bioluminescence assay. Light emission was detected with a Beckman scintillation counter using a single photomultiplier. 50 μl of the CLS reagent was diluted in 3.5 ml of reaction buffer (20 mM Heps, 10 mM MgCl₂, 2 mM EDTA, 0.18 mM DTT, and 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 the inhibition of the initial rate by excess AMP. Under these conditions, a range of 5 (10⁶ cpm)–40 pmol (10⁶ cpm) of ATP could be determined. ATP content is expressed as nmol/10⁶ cells.

**Determination of Adenylate Cyclase Activity**—Adenylate cyclase activity was determined in cell lysates by the method of Salomon et al. (21), which involves the separation of [32P]AMP from [32P]ATP on sequential columns of Dowex 50 and Alumina.

**RESULTS**

Incubation of 1321N1 cells with [125I]-EGF at 4 °C leads to selective binding to cell surface receptors (22). When such labeled cells are washed and the incubation is continued at 37 °C, in the presence or absence of labeled EGF, a rapid translocation of [125I]-EGF occurs from sites on the cell surface to sites within the cell (15). This translocation, which presumably occurs by receptor-mediated endocytosis, can be followed in either of two ways. Upon incubation at 37 °C of cells prelabeled at 4 °C with [125I]-EGF, the label rapidly moves from sites where the label is dissociated by acidic conditions (presumably cell surface sites) to sites where it is not dissociated (15). We also have found that, concomitantly, acid-stable [125I]-EGF appears in low density fractions of cell lysates upon centrifugation over sucrose gradients; whereas all acid-labile [125I]-EGF is associated with higher density fractions. The effect of PAO on this translocation has been investigated by combining both techniques. The centrifugation procedure was modified⁵ to substitute a step gradient for a continuous gradient of sucrose. The internalized (acid-stable) [125I]-EGF is collected at a 15/38% interface, while the cell surface (acid-labile) label accumulates at a 38/60% interface. Fig. 1 shows the time course of translocation of previously bound (4 °C) [125I]-EGF during a subsequent 37 °C incubation. Clearly, EGF bound at 4 °C is almost exclusively associated with the plasma membrane fraction (Fig. 1A), but rapidly moves into the low density fraction with time at 37 °C.

In a related experiment, the cells were washed at low pH to remove membrane-bound [125I]-EGF prior to lysis and centrifugation over sucrose gradients. The results (Fig. 1B) indicate that very little acid-stable [125I]-EGF is present immediately after binding at 4 °C. When the incubation was continued at 37 °C, acid-stable [125I]-EGF first appeared in association with the heavy fractions and shortly thereafter in the light fractions of the gradient.

In Fig. 2, the effect of PAO on binding and internalization of [125I]-EGF is illustrated. The protocol used is somewhat

³ J. Kurz, unpublished results.
Reversibility of the inhibition by PA0 of internalization of EGF

Cells were pretreated for 5 min with PA0 or 0.1% dimethyl sulfoxide in Hepses-Eagle's medium supplemented with 0.1% bovine serum albumin. 4 ng/ml (10^6 cpm/ml) of 125I-EGF was added and the cells were incubated either for 2 h at 4 °C or for 30 min at 37 °C. The cells were transferred on ice, washed, lysed, and layered on top of a sucrose step gradient. The 15/35% sucrose interface was collected and filtered, and radioactivity was determined.

| Pretreatment | 125I-EGF cpm | low density fraction |
|--------------|--------------|---------------------|
| Control      | 6647         |                     |
| 37 °C        | 4 °C         | 198                 |
| 100 µM PA0   | 196          |                     |
| 100 µM PA0, 1 mM glutathione | 336 |         |
| 100 µM PA0, 1 mM mercaptoethanol | 201 |         |
| 100 µM PA0, 1 mM DTT | 4179 |         |
| 100 µM PA0, 1 mM BAL | 4306 |         |

In the following experiments, cells were preincubated at 37 °C for 5 min with PA0 at a concentration of 100 µM. These conditions result in complete inhibition of receptor-mediated endocytosis even after extensive washing of the cells post-exposure. This is operationally important since, under the exposure conditions chosen, the complicating secondary actions of PA0 are readily reversed by washing as discussed below.

PA0 had been used by others to inhibit internalization of EGF (15) and other polypeptides (13, 14). PA0 is a trivalent arsenical and as such can form readily reversible bonds with single sulphydryl groups, or quite stable ring structures upon reaction with vicinal sulphydryl groups such as those in lipoic acid (23). In an attempt to characterize the chemical basis of inhibition of EGF endocytosis by PA0, the effects of a number of sulphydryl compounds were tested for their capacity to prevent or reverse its effects. The protocol was to incubate 1321N1 cells for 5 min at 37 °C with PA0 plus or minus a sulphydryl compound, then 125I-EGF was added, and the cells were incubated at 37 °C. At t = 10 min, either 1 mM DTT (0) or 1 mM BAL (A) was added. Cells were chilled at the time indicated, washed, and lysed, and vesicles (A) were separated from plasma membrane (B) by a sucrose step gradient.

![Figure 2](image-url)

**Fig. 2. Reversibility of the inhibition by PA0 of EGF internalization.** Cells were preincubated with (0) or without (A) 100 µM PA0 for 5 min at 37 °C. Then 4 ng/ml (10^6 cpm/ml) of 125I-EGF was added, and cells were incubated at 37 °C. At t = 10 min, either 1 mM DTT (0) or 1 mM BAL (A) was added. Cells were chilled at the time indicated, washed, and lysed, and vesicles (A) were separated from plasma membrane (B) by a sucrose step gradient.

![Figure 3](image-url)

**Fig. 3.** Differences in the scales of the y axes of Fig. 2, upper and lower panels, should be noted.

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ever, based on the results shown in Fig. 4, this mechanism seems not to offer a viable explanation for the actions of PAO. PAO has no effect on ATP levels in 1321N1 cells under conditions (Fig. 4B) where it completely prevents internalization of \(^{125}\)I-EGF (or BAR, see below). Treatment of cells in a nutrient-depleted medium with PAO caused a modest decline in ATP levels, but this was prevented by the monofunctional sulfhydryl compound glutathione (Fig. 4A) which does not prevent the inhibition of endocytosis by PAO (Table I).

One of the purposes of this study was to extend our comparison of the mechanisms subserving ligand-induced BAR internalization and receptor-mediated EGF internalization. To this end, the effects of PAO on BAR internalization have been determined. Numerous previous experiments (1-4) have been consistent with the idea that exposure of 1321N1 cells to isoproterenol results in the rapid translocation of cell surface BAR to a location within cytosolic vesicles. Such vesicles can be isolated in low density fractions of sucrose gradients. In addition, the BAR in such vesicles appears to be inaccessible to hydrophilic ligands such as CGP-12177 (4, 28).

These two properties have been used to quantitate endocytosis of BAR. Thus, we have measured the appearance of BAR in low density fractions of cell lysates after centrifugation over sucrose gradients, or we have measured changes in the apparent affinity of the hydrophilic ligand CGP-12177 as a competitor of \(^{125}\)I-CYP binding to cell lysates.

PAO (100 \(\mu M\)) was added to cells 5 min prior to a 20-min incubation with 1 \(\mu M\) isoproterenol. Complete inhibition of the formation of BAR with low affinity for CGP-12177 or of formation of the low density form of BAR was observed (compare Fig. 5). The apparent ID\(_{50}\) for PAO for a 5-min incubation at 37 °C was 30 \(\mu M\) for inhibition of BAR internalization as well as for EGF internalization. As was the case for EGF internalization, bifunctional but not monofunctional sulfhydryl compounds inhibited or reversed the effect of PAO on BAR internalization (Table II).

Initially our experiments with BAR were complicated by an effect of PAO on BAR function per se; that is to say, antagonist binding was reduced in agonist-pretreated cells (Table II). However, this effect of PAO was found to be reversed by extensive washing of the cells after the standard 5-min exposure to PAO. Such washing (5 \(\times\) 10 ml over 5 min) did not reverse inhibition of BAR internalization. Also, addi-

**Fig. 4. Effect of PAO on intracellular ATP concentration.**

A, cells were incubated in nutrient-depleted growth medium (○) and either 100 \(\mu M\) PAO (×) or PAO and 1 mM glutathione (○), or PAO and 1 mM DTT (□) was added. At the times indicated, cells were chilled and ATP was extracted. B, cells were incubated in nutrient containing Hepes Eagle’s medium (○) and either 100 \(\mu M\) PAO (×) or PAO and glutathione (○) or PAO and DTT (□) was added.

**Fig. 5. Reversible effect of PAO on internalization of BAR.**

Internalization of BAR was determined either as appearance of low affinity for CGP-12177 in lysates (left side) or as appearance of vesicular BAR or a sucrose step gradient (right side). Receptor distribution without pretreatment is shown in panel A for control (○, □) and desensitized (20 min with 1 \(\mu M\) isoproterenol) cells (○, □). Cyanopindolol binding is given as percent of binding in the absence of CGP-12177. BAR in the vesicular fraction was determined by the extent of binding in the presence of 50 \(\mu M\) \(^{125}\)I-CYP. Panel B shows the distribution of the receptors after pretreatment with 100 \(\mu M\) PAO and 1 mM glutathione for 5 min. Panel C shows the distribution of the receptor after pretreatment with 300 \(\mu M\) PAO and 1 mM DTT.
was determined in the presence or absence of 10 nM CGP-12177. Nonspecific binding was determined in the presence of 1 µM propranolol. The right column shows specific binding of \(^{125}\text{I}-\text{CYP}\). This value was defined as 100%. The left column shows binding of \(^{125}\text{I}-\text{CYP}\) in the absence of CGP-12177 (right column). Low percentage \(^{125}\text{I}-\text{CYP}\) binding is therefore due to high accessibility for CGP-12177, which means less receptor internalization.

Inhibition of formation of low affinity for CGP-12177

Cells were pretreated for 5 min at 37°C with 100 µM PAO. 1 µM isoproterenol together with the thiol reagents was added for an additional 20 min. Cells were lysed and binding of 10 pM \(^{125}\text{I}-\text{CYP}\) was determined in the presence or absence of 10 nM CGP-12177. Nonspecific binding was determined in the presence of 1 µM propranolol. The right column shows specific binding of \(^{125}\text{I}-\text{CYP}\). This value was defined as 100%. The left column shows binding of \(^{125}\text{I}-\text{CYP}\) in the presence of CGP-12177. This is expressed as percent of binding which means less receptor internalization.

| Pretreatment | \(B_{\text{max}}\) | \(K_d\) |
|-------------|-----------------|--------|
| 0.1% dimethyl sulfoxide | 7.6 | 0.4 |
| 0.1% dimethyl sulfoxide, 1 µM isoproterenol | 7.6 | 0.4 |
| 100 µM PAO | 7.6 | 0.4 |
| 100 µM PAO, 1 µM isoproterenol | 7.6 | 0.4 |
| 100 µM PAO, wash | 7.6 | 0.4 |
| 100 µM PAO, wash, isoproterenol | 7.6 | 0.4 |
| 100 µM PAO, 1 µM glutathione, 1 µM isoproterenol | 7.6 | 0.4 |

TABLE II

Effect of PAO pretreatment on antagonist binding

Cells were pretreated for 5 min with PAO, followed by a 20-min incubation with or without isoproterenol. In one set of experiments, cells were washed extensively before isoproterenol was added. In the experiment described in line 7, 1 µM glutathione was added together with isoproterenol. Cells were washed and lysed and analyzed according to Scatchard (33).

Discussion

Previous investigators have reported that PAO inhibits internalization of protein into oocytes (12) and receptor-mediated endocytosis of protein nexin (14), EGF (15), and insulin (13). However, the mechanism of action of this trivalent arsenical was not explored. Early studies (23) established that trivalent arsenicals form stable ring structures with molecules having appropriately spaced vicinal sulfhydryl groups. Five-membered rings appear to be most stabile. The equilibrium of such a reaction strongly favors the complex, and monofunctional sulfhydryl agents are not effective in competing in such a reaction (23). Thus, the results of our study are consistent with these earlier observations.
studies tentatively point to the involvement of vicinal sulphydryl groups in the inhibitory action of PAO on receptor-mediated endocytosis.

Wiley and Cunningham (15) stated that PAO caused an irreversible inhibition of EGF internalization in cultured human fibroblasts. It is a consistent finding that extensive washing of PAO-treated cells does not reinstate endocytosis. Irreversible inhibition could result from nonspecific interactions and in such a case PAO would be a less useful tool in the elucidation of the molecular mechanism of endocytosis. For example, trivalent arsenicals are known to interact with a large variety of enzymes including those involved in ATP generation. Thus, irreversible inhibition of ATP production could lead to general debilitation of cell integrity and a loss in the capacity to mount a complex event like endocytosis. Others have cautioned against the use of PAO-treated cells after 3–4 h (13).

Our studies provide two useful, probably generally applicable items of information. First, the effects of PAO on endocytosis can be prevented and, more importantly, reversed by bifunctional sulphydryl compounds. Second, PAO inhibits endocytosis under conditions where cellular ATP levels are not changed. The stability of the inhibited state and its reversal by bifunctional, but not monofunctional, sulphydryl agents suggests a somewhat selective interaction with a biological molecule containing vicinal sulphydryl groups.

One purpose of our studies was to examine further the similarities in the endocytosis of BAR and EGF. Preliminary work from our laboratory had shown that the time courses of internalization of BAR and EGF were similar (but not identical): both processes were blocked by reduced temperature, both were blocked by treatment of cells with concanavalin A (22), and both were blocked by a reduction in cellular ATP (although not to the same extent). The present study demonstrates that endocytosis of both BAR and EGF are inhibited in similar fashion by PAO. Furthermore, it is demonstrated that PAO blocks the appearance of acid-stable 125I-EGF in a plasma membrane fraction, suggesting that this form of the EGF-receptor complex is a consequence of endocytosis. The time course of its appearance (about 5 min earlier than in the low density fraction) suggests further that it occurs at an early step during endocytosis. A possibly related observation was recently made with BAR (3). In this case, isoproterenol induced a form of the BAR that was inaccessible to hydrophilic ligands, but that co-migrated with the heavy fraction on sucrose gradients. A possible explanation for both of these observations is the formation of invaginated segments of the plasma membrane that were yet to form as completed endo-

![Graph](image_url)

**FIG. 7. Effect of PAO on agonist binding.** Competition binding of 125I-CYP and isoproterenol was performed in lysates in the presence of either 100 μM PAO (○), PAO and 1 mM glutathione (Δ), PAO and 1 mM DTT (□), PAO and 500 μM GTP (○), or with 0.1% dimethyl sulfoxide (●). Cyanopindolol binding is shown as percent of binding in the absence of isoproterenol.

**TABLE IV**

| Pretreatment | Stimulation over basal activity by 10 μM isoproterenol 10 mM NaF |
|--------------|---------------------------------------------------------------|
| 0.1% dimethyl sulfoxide<sup>a</sup> | | |
| Control      | 12.5 ± 1.6 | 9.3 ± 1.8 |
| Desensitized | 8.9 ± 1.3  | 10.5 ± 2.1 |
| 100 μM PAO<sup>b</sup> | | |
| Control      | 5.8 ± 1.4  | 14.5 ± 1.0 |
| Desensitized | 3.6 ± 0.3  | 12.3 ± 0.7 |
| 100 μM PAC, 1 mM glutathione<sup>c</sup> | | |
| Control      | 14.0 ± 2.6 | 9.5 ± 1.1 |
| Desensitized | 9.1 ± 1.2  | 8.5 ± 3.2 |
| 100 μM PAO, 1 mM DTT<sup>d</sup> | | |
| Control      | 20.3 ± 4.0 | 16.4 ± 3.0 |
| Desensitized | 13.9 ± 1.9 | 16.5 ± 2.8 |
| 100 μM PAO, 1 mM BAL<sup>e</sup> | | |
| Control      | 27.2 ± 2.8 | 17.9 ± 2.0 |
| Desensitized | 17.0 ± 2.7 | 19.8 ± 3.0 |

<sup>a</sup>n = 4.
<sup>b</sup>n = 2.
<sup>c</sup>n = 3.
<sup>d</sup>n = 3.
<sup>e</sup>n = 4.
<sup>f</sup>n = 2.
some, as has been described in other systems (32).

Since PAO had secondary effects on BAR, it was necessary to develop conditions to separate its actions on BAR properties per se and effects on endocytosis. Fortunately, the former effects of PAO were completely prevented by glutathione or 2-mercaptoethanol. When these secondary effects of PAO were prevented, internalization of BAR remained inhibited; however, uncoupling between BAR and adenylate cyclase still occurred. Therefore, consistent with previous observations (19) it is possible to distinguish reactions leading to uncoupling and those leading to receptor internalization. It follows that 1321N1 cells possess a mechanism for uncoupling the cellular response to the ligand even when receptor internalization is prevented.

Ligand-induced endocytosis of BAR occurs in 1321N1 cells by a process we have yet to be able to distinguish from receptor-mediated endocytosis of EGF. However, our results only address similarities in the early steps of the overall process; thus, we cannot comment on possible similarities in steps subsequent to internalization such as recycling of receptor or ligand to the cell surface, or degradative pathways for receptor or ligand. However, to the extent that subsequent steps in the pathways can be shown to be similar, studies of BAR processing may be generally enlightening of the mechanism of endocytosis, since, of the common receptors studied in this area, only the BAR can be measured directly after internalization has occurred. The availability of a number of reagents, as has been described in other systems (32).

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