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Differentiation between Amine Transport and β-Adrenergic Receptor-Mediated Binding in Cultured Mammalian Cells

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SUMMARY

We have found that several types of cultured mammalian cells, including both normal and transformed human, rat, and mouse cell lines, have an active transport system for a diverse group of structurally related compounds possessing an amine group and various types of aromatic ring structures. Ligands such as the β-adrenergic antagonists (−)-[^{3}H]dihydroalprenolol (DHA), (−)-[^{3}H]propranolol, and (−)-[^{125}I]iodocyanopindolol, and the tricyclic antidepressant [^{3}H]imipramine, which are used to assess cell surface receptors for catecholamines and serotonin, appear to be actively transported into cells rather than simply bound to cell surface sites or accumulated by passive diffusion. DHA transport was competed by many structurally related amines including imipramine and certain α- and β-adrenergic ligands, but not by catecholamines or serotonin. Ligand transport in HeLa cells was saturable at micromolar levels, selective, nonstereospecific, temperature- and pH-dependent, and sensitive to the ionophore monensin and the amine transport inhibitor reserpine, thus indicating dependence on a carrier system driven by a transmembrane proton gradient. In C6 glioma cells, amine transport was clearly distinguishable from β-adrenergic receptor binding which could be measured with the recently developed hydrophilic β-blocker (±)-[^{3}H]4-(3-tertiarybutylamino-2-hydroxy-propoxy)-benzimidazole-2-on hydrochloride (CGP-12177); binding of this ligand met rigorous pharmacological criteria, was not influenced by monensin or reserpine, and, therefore, did not appear to be transported. Membrane vesicles from HeLa and C6 cells transported DHA but not CGP-12177 via a MgATP-dependent mechanism which was inhibited by *N,N'*-dicyclohexylcarbodiimide, monensin, and reserpine, indicating a carrier system driven by a proton gradient maintained by a proton-pumping ATPase.

INTRODUCTION

Catecholamines influence the metabolism of their target cells by binding to specific cell surface receptors and, in a process mediated by a family of transducing proteins, by regulating the catalytic subunit of adenylate cyclase (1). To better understand the regulation of coupling between receptor and adenylate cyclase, we have studied this process in cultured mammalian cells (Ref. 2 and references therein). Recent studies of the interaction of DHA,[1] ICYP, propranolol, and imipramine with C6 glioma and HeLa cells have led us to conclude that these compounds are taken up by cells via an active transport system for amines which we can clearly distinguish from authentic β-adrenergic receptor function. Previous studies with basic amines such as propranolol and imipramine have found uptake into perfused lung and macrophages (Ref. 3 and references therein) to be saturable, temperature sensitive, pH dependent, nonstereoselective, and sensitive to metabolic inhibitors as we find here for cultured cells. In those reports, however, the mechanism of accumulation was not defined. Our results indicate that uptake is not dependent on the pK_a, hydrophobicity, or diffusion of the compounds. Rather, our data implicate a proton gradient as the driving force for a carrier-mediated amine transport system.

[1] The abbreviations used are: DHA, (−)-[^{3}H]dihydroalprenolol; ICYP, (−)-[^{125}I]iodocyanopindolol; DCCD, *N,N'*-dicyclohexylcarbodiimide; CGP-12177, (±)-[^{3}H]4-(3-tertiarybutylamino-2-hydroxy-propoxy)-benzimidazole-2-on hydrochloride; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}-1-propanesulfonic acid.

MATERIALS AND METHODS

Cell lines. The HeLa cell lines ES-1 (HeLa Stone) and D98/AH-2 of epithelial morphology, the hybrids 444 and ESH5T, and the human fibroblast GM77 were kindly provided by Dr. Eric Stanbridge, University of California, Irvine. The hybrids 444 and ESH5T were derived in Dr. Stanbridge's laboratory from independent clones of the cross D98/AH-2 × GM77 (4). The nontumorigenic hybrid 444 has a morphology intermediate between its parents, while the tumorigenic hybrid ESH5T

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has an epithelial morphology. C6 rat glioma cells (CCL 107) and 3T3-L1 mouse fibroblasts (CCL 92.1) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. C6 glioma cells were received at passage 39 and were not used above passage 60. Regular testing for mycoplasma revealed no contamination in these cell lines.

Cell culture. Dulbecco's modified Eagle's minimum essential medium with high glucose (25 mM), nonessential amino acids, and L-glutamine (DMEM) was used to maintain the HeLa cells, hybrids, C6 glioma, and 3T3-L1 cell lines which were routinely grown in the presence of 5% FCS; GM77 cells were grown in DMEM plus 20% FCS. A serum-free chemically defined medium for HeLa cells based on the medium described by Barnes and Sato (5) was used where indicated. This medium, which we call CDM, consisted of a 1:1 mixture of Dulbecco's MEM and Ham's F-12 plus nonessential amino acids and L-glutamine, and was supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (20 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), selenous acid (4 ng/ml), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, 15 mM). The mixture of Dulbecco's MEM and Ham's F-12 containing nonessential amino acids and L-glutamine, when supplemented with 5% FCS instead of the five growth factors and HEPES, was called D/H. Culture media and serum were obtained from Quality Biological, Inc. (formerly HEM Research), Rockville, MD. Cells were grown at 37° in a humidified 5% CO_2 atmosphere and were routinely subcultured by trypsinization.

Chemicals. (−)-[*ring,propyl*-^{3}H]dihydroalprenolol(specific radioactivity, 90 or 104.8 Ci/mmol), (−)-[4-^{3}H]propranolol (specific radioactivity, 21.3 Ci/mmol), [*methyl*-^{3}H]imipramine (specific radioactivity, 70.7 Ci/mmol), 5-[1,2-^{3}H]serotonin (specific radioactivity, 28.5 Ci/mmol), and Aquasol were obtained from New England Nuclear. (±)-[^{3}H]CGP-12177 (specific radioactivity, 42.7 or 50.2 Ci/mmol) and (−)-[3-^{125}I]iodocyanopindolol (specific radioactivity about 2000 Ci/mmol) were obtained from Amersham Corp. MOPS (free acid and Na^+ salt), MES or TAPS, were from Research Organics, Inc., Cleveland, OH. RO20-1724 was a gift from Hoffman-La Roche. Propranolol (both (−)- and (+)-isomers) were gifts from Ayerst Laboratories, New York, NY. Phentolamine (Regitine HCl) was a gift from Ciba Pharmaceutical Corp. Unlabeled CGP-12177 was a gift from Ciba-Geigy Limited, Basel, Switzerland. Epidermal growth factor (culture grade) and selenous acid were obtained from Collaborative Research, Inc., Waltham, MA. All other chemicals were purchased from Sigma. Transferrin (T-2252), insulin (I-5500), and hydrocortisone (H-4001), all from Sigma, were used as growth factors in CDM.

Binding and uptake assays. Assay buffer consisted of NaCl (125 mM) buffered with MOPS (25 mM) and, when whole cells were assayed, contained either RO20-1724 (0.1 mM) or isobutylmethylxanthine (5 mM). MOPS was chosen as buffer because it has a pK_a of 7.2 at 20°, buffers our assay mixture adequately at both pH 6.2 and 8.2, and is relatively insensitive to temperature changes. MOPS also has low absorbance in the Lowry protein assay compared to HEPES (6). Comparable results were obtained in the DHA uptake assay when MES or TAPS was substituted for MOPS at pH 6.2 and 8.2, respectively. Most assays were performed at pH 8.2; exceptions are noted in the figure or table legends.

Cells were harvested by scraping from culture dishes with a rubber policeman into either growth medium or MOPS/NaCl assay buffer, washed with 5 ml of assay buffer, and resuspended in assay buffer. Cell viability was >90% for HeLa and >80% for C6 glioma when determined by trypan blue exclusion at the end of an assay. C6 glioma cells were added at 10×10^5 cells/tube, and all other cells were added at 2×10^5 cells/tube after cells were counted in a model ZH Coulter Counter equipped with a Coulter Channelizer. Cells were kept on ice after harvesting and incubated for 15 min on ice with the appropriate inhibitor when one was used. Monensin and reserpine were dissolved in 95% ethanol; the final ethanol concentration was less than 1% which had no effect on uptake or binding. Assays were performed in triplicate and initiated by addition of radiolabeled ligand. Optimum temperatures and times of incubation for equilibrium binding were determined for each ligand; assays with DHA, [^{3}H]imipramine, and [^{3}H]propranolol were incubated at 20° for 20 min, with CGP-12177 at 37° for 20 min, and with ICYP at 25° for 45 min unless otherwise noted. We found no evidence for ligand degradation when DHA was incubated with cells for 30 min at 37° and compared with authentic ligand by TLC. DHA and CGP-12177 were used at final concentrations of 2.5 or 5 nM; ICYP was used at a final concentration of 100 pM. The final assay volume was 0.5 ml in glass tubes for all ligands except ICYP, where the volume was 0.25 ml in polypropylene tubes. Binding or uptake was terminated with the addition of 7 ml of ice-cold 154 mM NaCl followed immediately by vacuum filtration of the tube contents through Whatman GF/B filters. Filters were rinsed twice with saline and removed from the manifold after 20 min of drying under vacuum (5 min of drying when CGP-12177 was the ligand), then transferred to glass vials containing 10 ml of Aquasol to count tritium in a liquid scintillation counter or to glass tubes for counting ^{125}I in a γ counter. Uptake was defined as the total cell-associated ligand minus the filter background. Background was less than 0.5% for DHA, CGP-12177, and [^{3}H]propranolol, and less than 1% for ICYP and [^{3}H]imipramine. Specific binding of CGP-12177 was defined as the total cell-associated ligand minus the amount bound in the presence of either 1 or 10 μM (−)-propranolol. For the preparation of partially purified membrane vesicles, cells were first allowed to swell for 10 min on ice in 1 mM MOPS (pH 8.2) containing 20 μM phenylmethylsulfonyl fluoride, then lysed by 10 strokes of a Dounce homogenizer equipped with a tight-fitting glass pestle. Homogenates were centrifuged at 890 × *g* to remove nuclei; supernatant fluid was then centrifuged at 30,000 × *g* for 20 min and the pellet resuspended in MOPS/NaCl assay buffer with a Brinkmann Polytron homogenizer set at speed 4 for 15 s. About 100 μg of membrane protein was added to each assay tube, and assays were performed as described for whole cells. Results are representative of experiments performed at least twice, with triplicate determinations varying less than 5% from the mean.

Protein was estimated by Hartree's modification (7) of the Lowry procedure with bovine serum albumin as standard.

RESULTS

Differentiation between Binding and Uptake

Ligand binding and uptake in HeLa cells. We have previously demonstrated β-adrenergic receptor function in HeLa cells (Ref. 2 and references therein). However, while assessing receptors on intact cells by ligand binding, we found that binding of the β-adrenergic antagonists DHA and ICYP to intact cells of HeLa strains D98/AH-2 and ES-1 did not conform to acceptable pharmacological criteria of specificity and stereoselectivity. Binding of DHA and ICYP was linear up to final concentrations of 40 nM and 500 pM, respectively (data not shown), which are far higher than the saturation values observed for the binding of these agents to β-adrenergic receptors (8). Furthermore, competition by propranolol was not stereoselective, and propranolol had 50% inhibitory concentrations (IC_{50}) of 1 and 10 μM for DHA and ICYP, respectively (Fig. 1, A and B). We concluded from these findings that these compounds were interacting with sites that were of lower affinity than typically seen for ligand binding to authentic β-adrenergic receptors. DHA binding was inhibited very effectively by the tricyclic antidepressant, imipramine, an inhibitor of serotonin transport in brain and platelets (9, 10) that does not bind to β-adrenergic receptors[2] but does inhibit propranolol uptake in macrophages (3). Finally, the α-ad-

[2] P. G. Lysko and R. C. Henneberry, manuscript in preparation.

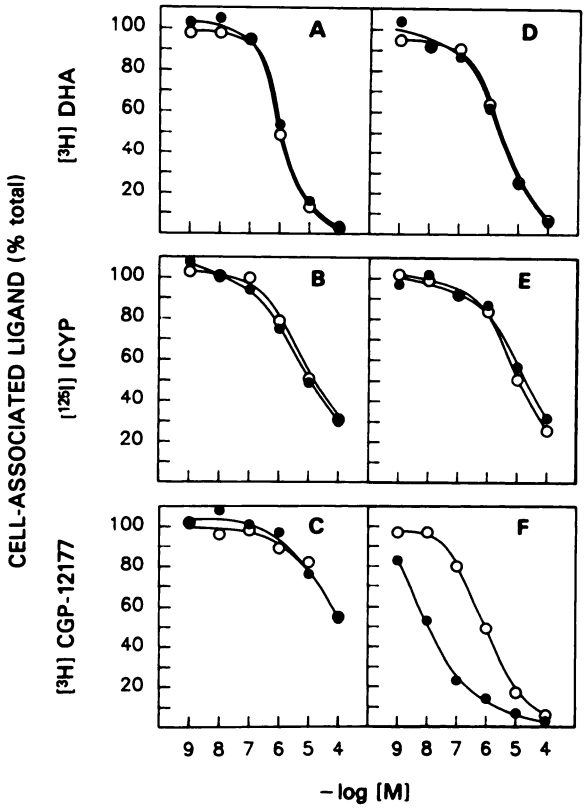

FIG. 1. *Comparison of two cell types and three β-adrenergic ligands for blocking by (−)- and (+)-propranolol*

The (−)- and (+)-isomers of propranolol were added to cell suspensions to block binding or uptake of radioligands. Cells were incubated with radioligand and increasing concentrations of competitor in assay buffer (pH 8.2) and rapidly filtered when equilibrium was reached. See "Materials and Methods" for details. Panels A, B, and C represent experiments with HeLa cells; panels D, E, and F represent experiments with C6 glioma cells. Panels A and D were with DHA (5 nM final concentration), B and E were with ICYP (100 pM), C and F with CGP-12177 at 5 and 2.5 nM, respectively. ●, competition by (−)-propranolol; ○, competition by (+)-propranolol. Results are expressed as the per cent of total cell-associated ligand in the absence of competitor. The 100% equivalencies are (fmol/mg of protein): A, 2286; B, 31; C, 28; D, 1122; E, 34; F, 60.

renergic antagonists phentolamine and yohimbine also displaced DHA (Table 1), although to a lesser extent than the β-adrenergic antagonists propranolol and alprenolol. Therefore, the interaction of the β-adrenergic ligands DHA and ICYP with HeLa cells did not seem to be mediated by an authentic β-adrenergic receptor. Rather, it appeared that these compounds were taken up by intact cells through independent sites or processes. Uptake of DHA was not through a "generalized amine uptake" process, since many amines failed to compete (Table 1). For example, neither serotonin, nor dopamine, nor any of the tested β-adrenergic agonists significantly blocked DHA uptake at concentrations below 100 μM where even lidocaine was slightly inhibitory. Epinephrine, norepinephrine, dopamine, and serotonin did not inhibit DHA uptake even when tropolone and iproniazid (0.1 mM each) were included to minimize the risk of degradation of these compounds by catechol-*O*-methyltransferase and monoamine oxidase, respectively. In addition, [^{3}H]serotonin failed to bind to or be transported into HeLa cells when incubated for 20 min at 20° at a final concentration of 5 nM.

TABLE 1

Competition of DHA uptake in HeLa cells by various compounds

Cells were grown in CDM and suspended for 20 min at 20° in assay buffer (pH 8.2) with 5 nM DHA and increasing concentrations (10^{-9}–10^{-4} M) of competitor as in Fig. 1.

Compound	IC_{50}[a]	Compound	IC_{50}
	μM		μM
(−)-Propranolol	1	(−)-Epinephrine[b]	≫100
(+)-Propranolol	1	(−)-Isoproterenol	≫100
(−)-Alprenolol	3	(−)-Norepinephrine[b]	≫100
Dichloroisoproterenol	1	(−)-Phenylephrine	≫100
Imipramine	0.3	Dopamine[b]	≫100
Yohimbine	21	Serotonin[b]	≫100
Phentolamine[c]	36	Lidocaine	≫100

[a] The inhibitory concentration competing 50% of the DHA bound in the absence of competitor.

[b] Incubated with or without 0.1 mM each tropolone and iproniazid.

[c] In addition, the α-adrenergic antagonists ergotamine and dihydroergotamine, and the α-adrenergic agonists clonidine and ephedrine were similarly found to inhibit DHA uptake.

Although HeLa cells could not be saturated with DHA at concentrations usually used for receptor-binding assays, saturation was achieved (Fig. 2) at around 2 μM final concentration, which is about 2 orders of magnitude greater than usually reported for β-adrenergic receptor binding (8). The apparent "K_m" for DHA uptake, estimated from a double-reciprocal plot of the data in Fig. 2, was 0.55 μM. If we had assumed that the amount of cell-associated ligand at saturation was entirely bound to the cell surface, approximately 42×10^6 receptors/cell would have been required, an extremely high number for any cell surface receptor.

Ligand binding and uptake in C6 glioma cells. The β-adrenergic antagonists DHA and ICYP appeared to bind to C6 glioma cells but, as with HeLa cells, propranolol competition of the binding of these ligands was not stereoselective (Fig. 1, D and E). These results suggested nonreceptor-mediated interaction of the ligands with the cells, possibly representing ligand uptake. Interaction of the partial agonist CGP-12177 with C6 cells appeared to represent binding to an authentic β-adrenergic receptor. This is illustrated in Fig. 1F by data showing that the (−)-isomer of propranolol competed for CGP-12177 binding to C6 cells with an IC_{50} of 13 nM, indicating the high affinity binding characteristic of β-adrenergic receptors; (+)-propranolol had an IC_{50} of more than 1 μM, nearly 2 orders of magnitude greater than that for (−)-propranolol. The binding of CGP-12177 to C6 glioma cells was complete by 20 min at 37° (Fig. 3A) and was saturable (Fig. 3B). A Scatchard plot of the binding data showed a K_D of 0.87 nM and a maximum number of receptors (B_{max})/cell of 9400; a plot of the same data by the method of Klotz (11) was sigmoidal (Fig. 3D) and gave the same number of receptors/cell as the Scatchard plot. These values are comparable to those reported for C6 glioma cells by Staehelin *et al.* (12), who introduced the use of CGP-12177 for binding studies. Since the binding of CGP-12177 to C6 glioma cells represented

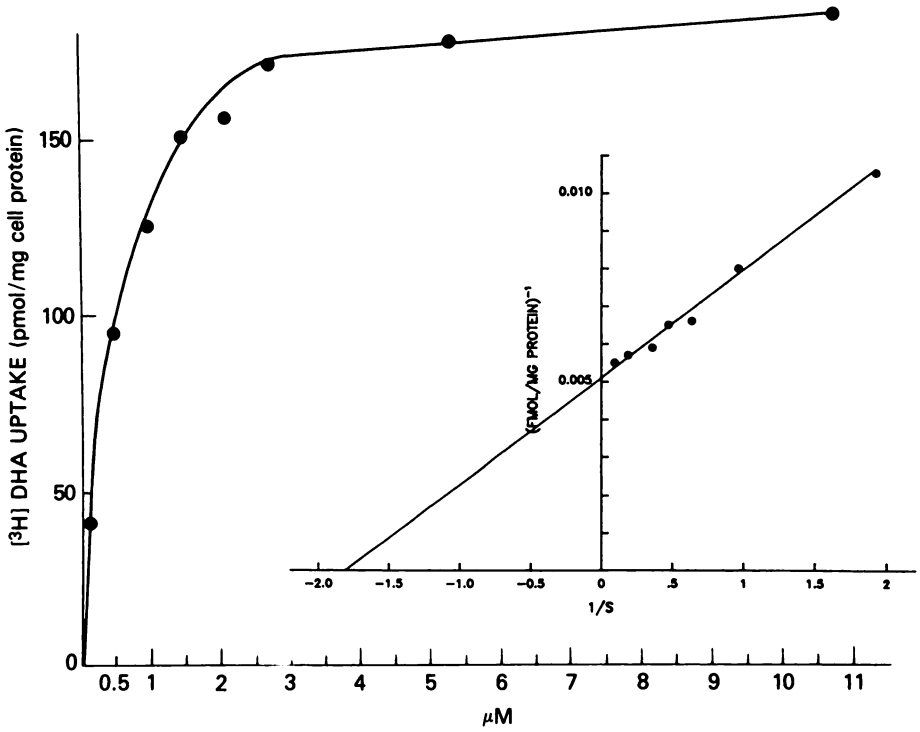

FIG. 2. *Saturation of DHA uptake in HeLa D98/AH-2 cells*

DHA was diluted to 0.1 Ci/mmol with unlabeled (−)-alprenolol. Cells were grown in CDM and incubated for 20 min at 20° in assay buffer (pH 8.2) with increasing concentrations of DHA. Uptake represents total cell-associated ligand minus filter background. The double-reciprocal plot was fitted by linear regression analysis and the apparent "K_m" was 0.55 μM.

binding to an authentic β-adrenergic receptor, whereas DHA and ICYP were taken up by intact C6 cells as well as by HeLa, it would appear that β-adrenergic receptors coexist on the same cell with distinct selective amine uptake sites. A major conclusion from these studies with C6 glioma cells is that CGP-12177 could be used to distinguish receptor binding from amine uptake.

Lack of correlation of uptake with lipophilicity or pK_a. The lipophilic nature of DHA has been suggested as the cause of high nonspecific binding in assays for β-adrenergic receptors (Ref. 12 and references therein). As shown in Table 2, we measured buffer:octanol partitioning at three pH values for four radiolabeled ligands used in these studies. Although CGP-12177 was the least lipophilic of the compounds tested, its partitioning into the octanol phase increased about 8-fold with increasing pH, a result comparable to the 11-fold increase for DHA. In contrast, the partitioning of ICYP into the octanol phase only increased about 2-fold over the pH range studied, yet ICYP as well as DHA was taken up by intact cells (Fig. 1). Like DHA, ICYP uptake was also pH dependent and blocked by monensin, whereas CGP-12177 binding was only slightly dependent on the assay pH and was not inhibited by monensin (Table 3). These results seemed to indicate a proton gradient rather than lipophilicity as a driving force for accumulation of ligand.

[^{3}H]Imipramine partitioning into the octanol phase only increased about 1.4-fold over the pH range tested (Table 2), yet imipramine was a potent competitor of DHA uptake (Table 1). Imipramine uptake was also pH dependent and inhibited by monensin.[2] As shown in Fig. 4, unlabeled alprenolol blocked both DHA and [^{3}H]imipramine uptake to the same extent. Similarly, unlabeled imipramine blocked both DHA and [^{3}H]imipramine uptake to the same extent, although at an apparently higher affinity than alprenolol. Thus, alprenolol and imipramine appeared to be interacting with a common site despite the wide differences in buffer:octanol partitioning between these compounds.

Imipramine (pK_a = 9.5), propranolol (pK_a = 9.45), and alprenolol (pK_a = 9.63) have nearly identical pK_a values, yet cells have higher affinity for imipramine than for DHA (Table 1, Fig. 4). A large percentage (40%) of the total added imipramine is found cell associated,[2] in contrast with the percentages (6–15%) noted for ICYP, [^{3}H]propranolol, and DHA (Table 3); only 0.5% of the total CGP-12177 (pK_a = 9.4,11.6) added became cell associated. Furthermore, reserpine has a markedly lower pK_a of 6.6, yet is an effective inhibitor of DHA uptake (Fig. 6) and is competitive as well.[2] Therefore, we conclude that neither lipophilicity nor pK_a is of major importance for the uptake of these ligands into intact cells.

Characterization of Amine Transport in Intact Cells and Membranes

Inhibitors of ligand transport in HeLa and C6 cells. The uptake of DHA into intact HeLa cells and membrane preparations was found to be markedly influenced by the pH of the assay buffer (Fig. 5, Table 3). Since external pH has been shown to be directly related to catecholamine transport and proton gradients in chromaffin granules (13, 14), we considered our results indicative of uptake mediated by a pH gradient across the plasma membrane. In Fig. 5, DHA uptake was about 3 pmol/mg

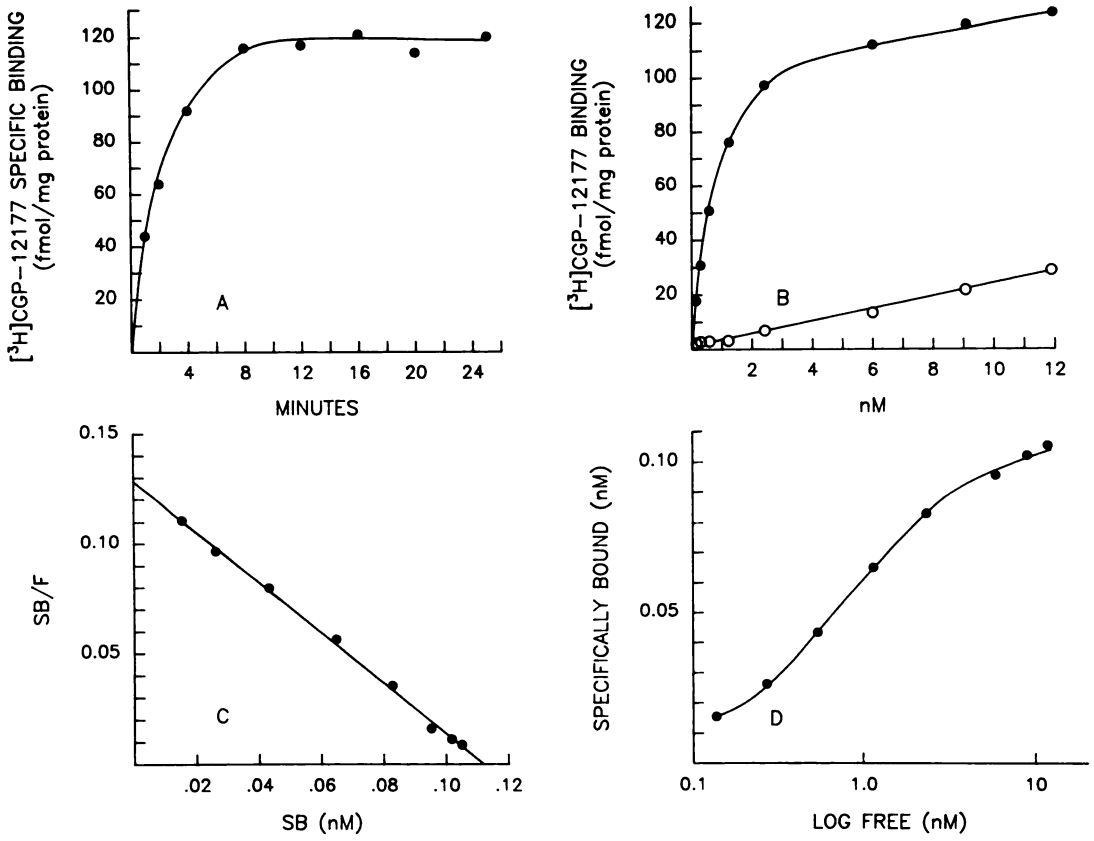

FIG. 3. *Binding of CGP-12177 to intact C6 glioma cells*

A. Specific binding *versus* time at 37° with 5 nM CGP-12177. Cells were suspended in assay buffer (pH 8.2) and incubated with CGP-12177 for varying lengths of time. B. Saturation of CGP-12177 binding. Cells were incubated in assay buffer (pH 8.2) for 20 min at 37° with increasing concentrations of CGP-12177. ●, specific binding; ○, nonspecific binding. C. Scatchard plot of binding data; K_D = 0.87 nM, B_{max} = 131 fmol/mg of protein or about 9420 receptors/cell. D. Binding data plotted according to Klotz (11). Inflection point indicates 0.5 B_{max}. Results are expressed as specific binding which equals total cell-associated ligand minus the amount bound in the presence of 10 μM (−)-propranolol (nonspecific binding). Nonspecific binding did not exceed 20% of total binding.

TABLE 2

Partitioning of radioligands into octanol/buffer at three pH values

Radioligands were added to MOPS/NaCl assay buffer at each pH and mixed with an equal volume of octanol, incubated at 20° for 20 min, and remixed every 5 min. Partitioning into each phase was determined by counting portions of both.

Radioligand	Octanol/buffer distribution		
	pH 6.2	pH 7.2	pH 8.2
DHA	5.7	11.6	61
ICYP	19	24	35
Imipramine	6.8	8.7	9.5
CGP-12177	0.11	0.16	0.83

of protein at pH 8.2. From dextran/H_2O ratios obtained during methylamine partitioning[3] we calculated an intracellular volume of 3.5 μl/2.5 × 10^6 cells. Protein estimations gave 2.5 × 10^6 cells/mg of protein, which calculated to 3 pmol accumulated in 3.5 μl, or 0.857 μM, a 340-fold concentration over the extracellular concentration. The Na^+/H^+ ionophore monensin, which dissipates proton gradients, was tested and found to inhibit DHA uptake into intact cells in a dose-dependent fashion (Fig. 6). Inhibition by monensin was nearly complete at 10 μM, indicating that the overwhelming majority of the DHA was transported into the cells rather than bound to receptors on the surface. In addition, the amine transport inhibitor reserpine, a potent inhibitor of carrier-mediated transport in chromaffin granules, synaptic vesicles, and platelet storage organelles (9, 10, 15, 16), also inhibited DHA uptake into intact HeLa cells in a dose-dependent manner (Fig. 6), indicating carrier-mediated transport. Table 4 shows that the uptake of DHA into C6 cells was inhibited by both the ionophore monensin and the amine transport inhibitor reserpine, but not by unlabeled CGP-12177, which should have displaced DHA bound to β-adrenergic receptors. In contrast, neither monensin nor reserpine inhibited the binding of CGP-12177 to C6 glioma cell β-adrenergic receptors, whereas unlabeled CGP-12177 was very effective as a competitor. By these criteria, ligand binding is clearly separable from ligand transport.

Inhibition of DHA uptake into HeLa and C6 cells by reserpine indicated carrier-mediated transport, which was also suggested when ligand exchange diffusion, initiated by dilution, was accelerated by addition of unlabeled ligand to the diluent (Table 5). The counterflow

[3] P. G. Lysko and R. C. Henneberry, submitted for publication.

TABLE 3

pH-dependent monensin-inhibitable DHA uptake by several cell lines

Cells were grown in DMEM unless otherwise noted and suspended in buffer at three pH values with 2.5 nM DHA (or another ligand where indicated). See "Materials and Methods" for details of each ligand use.

Cell line[a]	pH 6.2	pH 7.2	pH 8.2		
	fmol/mg protein				
ES-1	53	227	576	(93)[b]	[8][c]
D98/AH-2	82	449	880	(91)	[6]
D98/AH-2 (ICYP)[d]	6	21	50	(83)	[13]
D98/AH-2 (propranolol)[e]	319	1,985	11,409	(96)	[15]
GM77	339	1,682	4,161	(90)	[11]
444	134	810	2,328	(93)	[6]
ESH5T	114	544	1,089	(95)	[8]
3T3-L1	143	1,066	2,426	(81)	[10]
C6	89	432	982	(95)	[14]
C6 (CGP-12177)[f]	91	123	135	(0)	[0.5]

[a] Cell lines: ES-1 and D98/AH-2 are HeLa cells, D98/AH-2 and the human fibroblast GM77 are parents of the hybrids 444 and ESH5T, 3T3-L1 is a mouse fibroblast, and C6 is a rat glioma cell. See "Materials and Methods" for sources of cells.

[b] Per cent inhibition by 10 μM monensin (pH 8.2).

[c] Per cent of added ligand found cell associated at pH 8.2.

[d] ICYP (100 pM) instead of DHA, cells grown in CDM.

[e] [^{3}H]Propranolol (10 nM) instead of DHA, cells grown in CDM.

[f] CGP-12177 (7 nM) instead of DHA.

phenomenon of accelerative exchange diffusion has been interpreted as strong evidence for mobility of the carrier site (17).

MgATP dependence of DHA transport in membranes. Amine transport into chromaffin granules (Refs. 13, 18, and references therein), synaptic vesicles (19), and platelet storage organelles (10, 20) has been reported to depend on both Mg^{2+} and ATP. DHA uptake in membrane preparations of both HeLa and C6 showed a striking dependence on MgATP concentration (Figs. 7 and 8). In contrast, CGP-12177 binding to C6 membranes was independent of MgATP (Fig. 8). Therefore, use of the two ligands permitted us to distinguish clearly between β-adrenergic receptor binding and amine transport in membrane preparations of C6 glioma cells, as we showed above for intact cells. Furthermore, monensin, reserpine, and the ATPase inhibitor DCCD inhibited the MgATP-dependent uptake of DHA into HeLa (Fig. 9) and C6 (data not shown) membrane preparations. When the effect of DCCD was titrated, 50 μM was as effective as 500 μM, with an IC_{50} of about 13 μM or 50 nmol/mg protein. This high a level of DCCD has been found necessary to inhibit the chromaffin granule proton-pumping ATPase (21). Although we assumed vesicle formation in these membranes due to the nature of the preparative procedure, the actions of monensin and DCCD to abolish proton gradient formation was functional proof of vesicular uptake. We believe the functioning vesicles to be inside out, with the proton-pumping ATPase on the outside pumping protons inward to generate a ΔpH (acid inside). In the presence of the above inhibitors, ligand association was the same as without ATP, indicating that these inhibitors blocked vesicular uptake but not surface binding. The demonstration of MgATP-dependent uptake in membrane vesicles rules out the possibility of receptor-mediated endocytosis of ligand, which would not function in a disrupted cell preparation.

Temperature dependence of transport. Attempts to measure rates of transport of ICYP into intact HeLa cells were frustrated by ligand efflux with time, especially at higher temperatures (Fig. 10). Efflux was probably

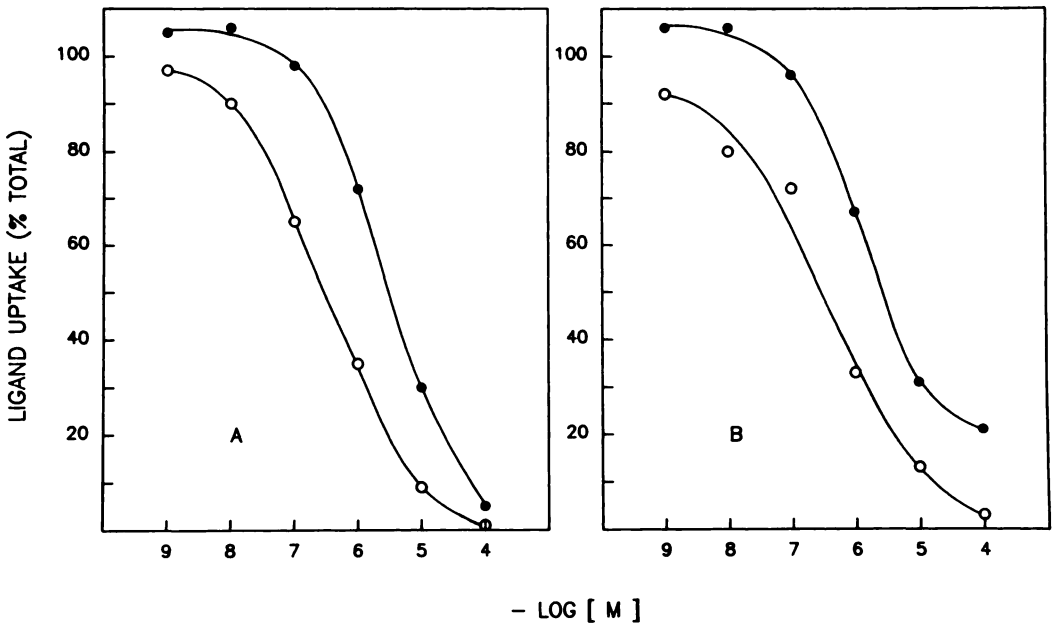

FIG. 4. *Comparison of imipramine and alprenolol competition*

HeLa ES-1 cells were grown in CDM and incubated for 20 min at 20° in assay buffer (pH 8.2) in the presence of 2.5 nM DHA (A) or 2.5 nM [^{3}H]imipramine (B) plus increasing concentrations of unlabeled (−)-alprenolol (●) or unlabeled imipramine (○). The 100% equivalencies are (fmol/mg protein): A, 262; B, 611.

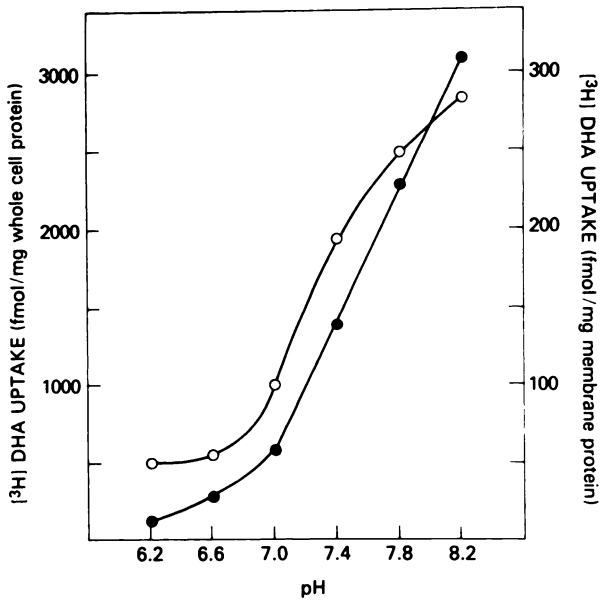

FIG. 5. *pH dependence of DHA uptake in HeLa D98/AH-2 cells*
Cells were grown in CDM. Intact cells and membranes were washed and assayed at each pH as described under "Materials and Methods." Final concentration of DHA was 2.5 nM for intact cells (●) and 5 nM for membrane preparations (○). Results are expressed as total associated ligand minus the filter background.

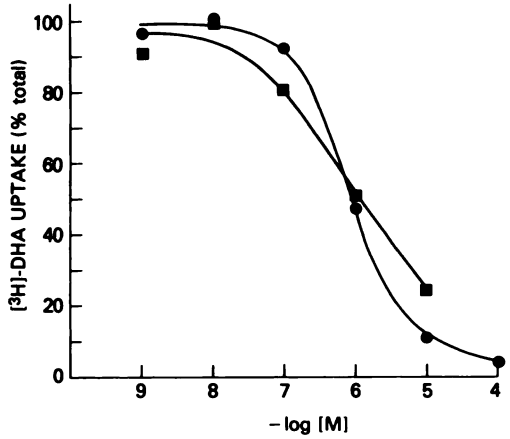

FIG. 6. *Monensin and reserpine inhibition of DHA uptake into HeLa D98/AH-2 cells*
Cells were grown in CDM and incubated for 20 min at 20° in assay buffer (pH 8.2) with 2.5 nM DHA in the presence of increasing concentrations of the inhibitors monensin (●) or reserpine (■). Results are expressed as per cent of total uptake in the absence of inhibitors; 100% is equivalent to 2014 fmol/mg of protein.

due to the breakdown of the membrane potential of cells resuspended and assayed in buffer. This same phenomenon has been noted for catecholamine transport in synaptic vesicles (19) and chromaffin granule membrane vesicles (22), where efflux is thought to reflect the transient nature of the ΔpH which is maintained by ATP hydrolysis. Accordingly, when cells were washed, resuspended, and assayed with growth medium present they continued to accumulate ligand, presumably due to their higher level of metabolic activity and ability to maintain a membrane potential (Fig. 10). The biphasic nature of uptake at 20° and at 37° in the presence of growth

TABLE 4

C6 glioma cells: inhibitors of DHA uptake do not affect CGP-12177 binding

Cells were grown in DMEM and incubated for 20 min at 37° in assay buffer (pH 8.2) with either DHA or [^{3}H]CGP-12177, in the presence or absence of inhibitor or unlabeled CGP-12177

Additions	Cell-associated ligand	
	DHA[a]	[^{3}H]CGP-12177[b]
	fmol/mg protein	
None	717	98
Reserpine (10 μM)	282	98
Monensin (10 μM)	79	99
CGP-12177 (100 μM)	716	5

[a] Final concentration was 2.5 nM. Cell-associated ligand was calculated as total associated ligand minus the filter background.

[b] Final concentration was 5 nM. Cell-associated ligand was calculated as total associated ligand minus the amount bound in the presence of 1 μM (−)-propranolol. Nonspecific binding was about 15%.

TABLE 5

Exchange diffusion in HeLa cells

Cells were grown in CDM and incubated for 20 min at 20° in assay buffer (pH 8.2) with 2 nM DHA or 2.5 nM [^{3}H]imipramine. The reaction mixture was diluted 10-fold into assay buffer or into buffer containing 1000-fold excess of unlabeled ligand. Efflux was measured over a 2-min time period, and the time for dissociation of half the radioligand ($t_{1/2}$) was estimated from the efflux curves.

Radioligand	$t_{1/2}$	
	Buffer alone	Buffer plus ligand
	sec	
DHA	96	36
[^{3}H]Imipramine	44	17

medium (Fig. 10) is not understood at this time. This problem will likely be resolved by the use of an alternative cell line lacking β-adrenergic receptors or an alternative ligand which does not bind to β-adrenergic receptors. Nevertheless, the temperature dependency of uptake is apparent in Fig. 10, with rates for the initial 10 min having temperature coefficients (Q_{10}) much greater than 2, indicative of transport rather than simple diffusion. Although the complete curve is not shown in Fig. 10, cells incubated at 37° with growth medium accumulated 74 fmol of ICYP/mg of protein by 75 min, at a linear rate of 0.48 fmol/mg of protein/min from 30 min.

Survey of several cell lines for amine transport. Several cell lines currently in use in this laboratory were examined and appeared to possess a system for the transport of amines (Table 3). The criteria used to identify transport were pH dependency and monensin inhibition of association of ligand with cells. Both normal and tumor cell lines of three species (human, mouse, and rat) were represented. All seven of the cell lines tested showed pH-dependent monensin-inhibitable DHA uptake. The fibroblasts GM77 and 3T3-L1 and the hybrid 444 were especially active in taking up DHA. HeLa strain D98/AH-2 also appeared to take up ICYP and [^{3}H]propranolol by the same mechanism. These results suggest that this transport system for amines may be ubiquitous and metabolically important for many cell types.

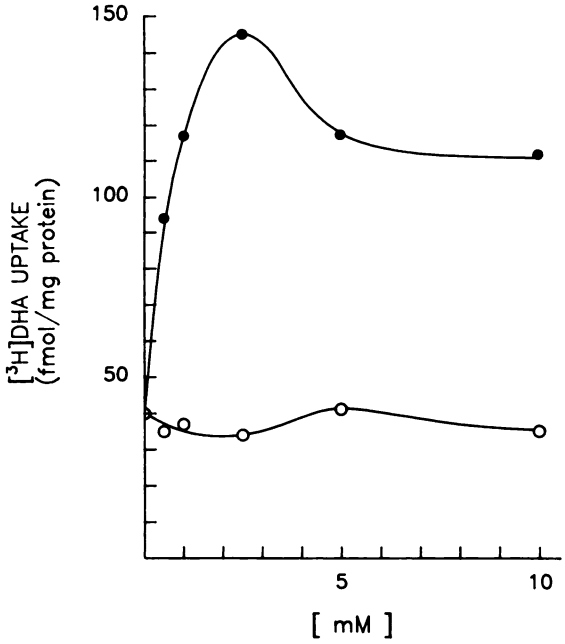

FIG. 7. *MgATP dependence of DHA uptake in HeLa ES-1 membrane vesicles*

Cells were grown in CDM, and membrane vesicles were prepared by hypotonic lysis as described under "Materials and Methods," then incubated for 20 min at 20° in assay buffer (pH 8.2) with 2.5 nM DHA in the presence of increasing concentrations of $MgCl_2$ (O) or $MgCl_2$ plus ATP (●). Results are expressed as total associated ligand minus the filter background.

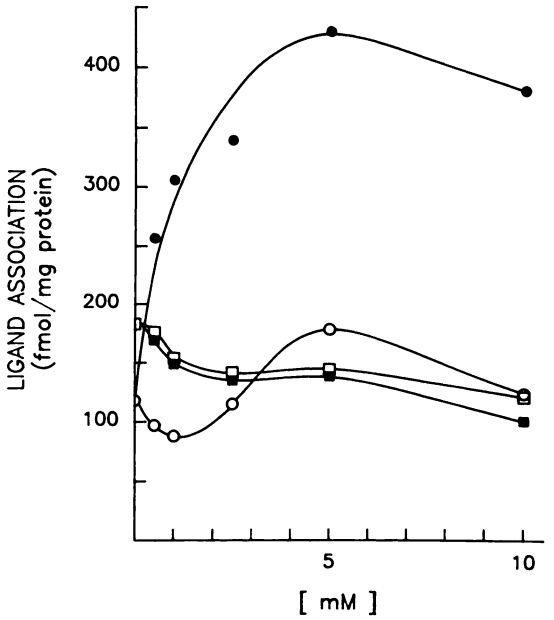

FIG. 8. *MgATP dependency of DHA uptake but not of CGP-12177 binding in C6 glioma cell membrane vesicles*

Cells were grown in CDM, and membrane vesicles were prepared by hypotonic lysis as described under "Materials and Methods," then incubated for 20 min at 20° with 2.5 nM DHA (circles) or CGP-12177 (squares) in assay buffer (pH 8.2) containing increasing concentrations of either $MgCl_2$ (open symbols) or $MgCl_2$ plus ATP (closed symbols). Results are expressed as total associated ligand minus the filter background.

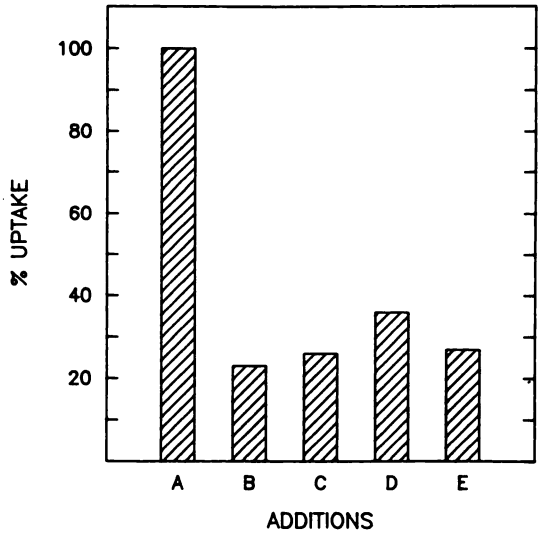

FIG. 9. *Inhibition of DHA uptake in HeLa ES-1 membrane vesicles*

Cells were grown in CDM and membrane vesicles prepared and assayed as in Fig. 7. $MgCl_2$ and ATP were present at 2.5 mM except as indicated. A. No further additions. B. ATP omitted. C. 10 μM monensin added. D. 10 μM reserpine added. E. 500 μM DCCD added. Results are expressed as per cent of total uptake in the presence of 2.5 mM MgATP minus the filter background; 100% is equivalent to 143 fmol/mg of protein.

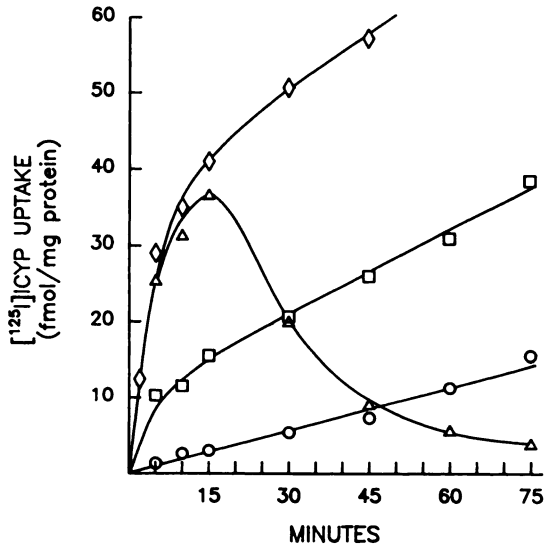

FIG. 10. *Temperature dependency of ICYP uptake in HeLa D98/AH-2*

Cells were grown in CDM and incubated at pH 8.2 in assay buffer or buffer plus growth medium (40:60) with 100 pM ICYP for varying lengths of time at different temperatures. O, 4° in buffer; □, 20° in buffer; Δ, 37° in buffer; and ◇, 37° in buffer plus growth medium. Results are expressed as total cell-associated ligand minus the filter background.

DISCUSSION

The inability of DHA and ICYP to exhibit saturable stereospecific binding to either HeLa cells or C6 glioma cells, both of which can be shown to contain β-adrenergic receptors by other criteria, indicates that not all radioligands used to quantify β-adrenergic receptors are suitable for all cell types. Numerous binding studies have been

performed with intact cells but not all have provided unequivocal pharmacological evidence that only β-adrenergic receptors are measured. Problems with high nonspecific binding attributable to ligand uptake by intact cells have been mentioned previously by Williams and Lefkowitz (8). However, receptors on intact frog erythrocytes were reported to bind DHA (23) in essentially the same fashion as receptors in membrane preparations; all relevant pharmacological criteria appear to have been fulfilled in this case. In many other instances using other intact cells and/or different β-adrenergic ligands, pharmacological criteria have not been so clearly met. Often, assay conditions have been manipulated to deal with high "nonspecific binding." Such manipulations have included: (*a*) the addition of the α-adrenergic antagonist phentolamine to the assay buffer (24, 25); (*b*) the addition of propranolol or phentolamine to the washing buffer (25, 27); (*c*) extensive washing (24, 25, 27); or (*d*) the use of an excessively high concentration of propranolol relative to radioligand concentration for determining the specific component of binding (26, 27). We mention these studies not to refute these authors' claims but to emphasize the inherent problems of receptor assessment on intact cells, particularly in the presence of previously unidentified amine transport sites. In some of these studies, the presence of two ligand-binding sites was inferred from radioligand dissociation or competition curves (24, 26, 28). Our results suggest that the lower affinity sites may actually represent radioligand uptake by an amine transporter. Indeed, the rapidly dissociable "nonspecific" component of (−)-[^{125}I]iodopindolol binding to C6-2B cells (27) closely resembles our results for DHA accelerative exchange diffusion. The speed of exchange diffusion plus the fact that both imipramine and alprenolol accelerated the process about 2.5-fold suggest independence from receptors. Re-examination of earlier work in the light of this information may help to resolve conflicts of receptor number determination (29) as well as discrepancies in K_D values between agonists and antagonists (12, 25, 26). Furthermore, our demonstration of competition for DHA uptake by the α-adrenergic antagonist phentolamine may explain its ability to displace [^{125}I]iodohydroxybenzylpindolol from putative receptors in other cell lines (25, 28).

CGP-12177 may not be the definitive β-adrenergic ligand for all cell types, since HeLa cells did not bind it very well. However, we found no evidence for CGP-12177 transport, indicating that this ligand will be useful to selectively evaluate β-adrenergic receptors in the presence of the amine transport system. Other purported β-adrenergic specific radioligands may still be useful, but if binding is largely pH dependent and inhibitable by monensin and reserpine, the possibility of ligand transport should be considered.

Inderal (propranolol hydrochloride) is the most frequently prescribed brand name pharmaceutical in the United States today (American Home Products Corp., 1983 Annual Report) with multiple indications besides its use as a cardiac blocker. [^{3}H]Propranolol was taken up by HeLa cells in a pH-dependent monensin-inhibitable manner, and unlabeled propranolol nonstereoselectively inhibited DHA and ICYP uptake, suggesting that transport may play a role in some of the therapeutic actions or possible deleterious side effects of this amine. Similarly, imipramine inhibition of DHA uptake, which was not inhibited by serotonin, suggests that some actions of this antidepressant (Tofranil) may be mediated by an amine transporter distinct from the platelet membrane serotonin transporter which is Na^+ dependent and inhibited by propranolol (30) and imipramine (9, 10) but not by reserpine (9, 10).

From the data presented in this paper we conclude that: (*a*) the elevated "nonspecific" component of β-adrenergic antagonist binding to intact cells is due to uptake of ligand by an active transport system for amines and not due to simple diffusion; (*b*) this amine transport system is saturable and selective for amines lacking aromatic ring hydroxyl groups; and (*c*) transport is dependent on a transmembrane pH gradient maintained by the hydrolysis of ATP and appears to be carrier mediated. Transport of DHA into HeLa cells was saturable, selective, temperature dependent, monensin and reserpine sensitive, and of equal or higher apparent affinity compared to other amine transport systems (18, 19). Since catecholamines and serotonin were not transported by the HeLa system, we refer to it simply as an amine transport system. All the compounds which competed DHA uptake in Table 1 were structurally similar amines, and it is reasonable to expect that such compounds would be transported as well. Indeed, we have identified a variety of amines which do or do not compete at this site. The minimum requirements for competition are an amino nitrogen linked by at least one carbon to an aromatic ring containing no hydroxyls.[2] The apparent exclusion of catecholamines, the nonstereoselectivity of competition by propranolol, and the failure of serotonin to be taken up distinguish the amine transport system of HeLa cells from chromaffin granule and synaptic vesicle amine transport, where norepinephrine, epinephrine, and dopamine are the normally transported substrates of a broadly specific transporter with greater affinity for (−)-isomers (18). However, the energetics of transport in HeLa appear to be the same as in the previously described systems. Amine transport into chromaffin granules, synaptic vesicles, and platelet storage organelles is coupled to a transmembrane proton gradient maintained by a proton-translocating Mg^{2+}-activated ATPase (10, 13, 14, 18–22, 31, and references therein). In these systems, as we have shown here for DHA uptake by intact cells and membrane preparations of HeLa and C6 glioma, transport is dependent upon the maintenance of a pH gradient which is sensitive to ionophores such as monensin. In regard to the action of monensin on DHA transport in HeLa, if monensin had initiated ion fluxes to stimulate the Na^+,K^+-ATPase, its effect should be abrogated by ouabain. Since there is no less inhibition by monensin when cells are incubated with 1 mM ouabain for 30 min prior to assay (data not shown), we judge the action of monensin to be independent of the Na^+/K^+ pump. Furthermore, in the above systems as we have shown here for DHA uptake by HeLa and C6 membrane vesicles, amine transport is driven by

Mg^{2+}-dependent ATP hydrolysis, is sensitive to the carrier-mediated transport inhibitor reserpine, and is sensitive to the ATPase inhibitor DCCD. The high concentrations of DCCD necessary to inhibit DHA uptake in HeLa correlate with the high levels needed to inhibit the chromaffin granule ATPase (21). These observations strongly suggest a common coupling mechanism for amine transport in these divergent systems.

Our description of this amine transport system in cultured cells poses a host of intriguing questions and should help to resolve some problems in the field of cell surface biochemistry. The relationship of amine transport to the bioenergetics of receptor-mediated endocytosis is also of interest in its own right. Preliminary evidence indicates that amine transport in HeLa is localized at the plasma membrane and is sensitive to inhibition by DCCD and *N*-ethylmaleimide but resistant to oligomycin. Such is also the case for the recently reported proton-pumping ATPase from clathrin-coated vesicles (32), which provides a precedent for the driving force behind our amine transport system.

As the preparation of this report neared completion, we found a somewhat similar report describing DHA uptake into HeLa cells (33). However, important differences between that report and our results include our demonstration of saturation, accelerative exchange diffusion, temperature-dependent efflux, the necessity for a proton gradient, and the clear differentiation between binding and uptake in both whole cells and membrane vesicles. Since our data also show the role of MgATP in generating a proton gradient in vesicles, we arrive at the markedly different conclusion that amine ligands are transported by a carrier-mediated process driven by a transmembrane proton gradient.

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