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TRANSPORT OF BETA-ADRENERGIC ANTAGONISTS IN THE ABSENCE OF BETA-ADRENERGIC RECEPTORS IN RAT PITUITARY TUMOR CELLS

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Abstract—We have demonstrated that the rat pituitary tumor cell line GH_3 has a carrier-mediated active transport system for the beta-adrenergic antagonist dihydroalprenolol (DHA). Transport of DHA in GH_3 was saturable, with an apparent K_m of 1.4 μM, was temperature and pH dependent, and was inhibited by the ionophore monensin and the amine transport inhibitor reserpine. Propranolol competed for DHA transport, but not in a stereoselective fashion. The tricyclic antidepressant imipramine also competed for DHA transport, but catecholamines or serotonin did not. This amine transport system in GH_3 cells appeared to be identical to the one we recently described in several other cell types; however, analysis in those cells was complicated by the fact that they contain beta-adrenergic receptors which bind beta-adrenergic ligands. In this report we show that GH_3 cells do not possess detectable beta-adrenergic receptors, based on their inability to bind the partial agonist CGP-12177, their inability to bind nanomolar concentrations of DHA in a saturable, stereospecific manner, and their failure to produce cAMP in response to stimulation by beta-adrenergic agonists. Characterization of the amine transport system in GH_3 cells clearly distinguishes it from receptor-mediated phenomena and should facilitate our efforts to fully understand its mechanism and significance.

The GH_3 cell line is a clonal derivative of rat pituitary tumor cells which has maintained several organ-specific functions during long-term *in vitro* culture [1, 2]. GH_3 cells have been shown to have specific receptors for a variety of hormones [3–5], and have provided useful models for the study of regulation of cell growth [6–8] and control of gene expression [9–11] by a variety of physiological and pharmacological agents. GH_3 cells have, for example, been useful in the demonstration of roles for Ca^{2+} and calmodulin in the regulation of prolactin gene expression [12].

We have found recently that many mammalian cells contain an active transport system for a group of basic amines which includes beta-adrenergic antagonists and tricyclic antidepressants; catecholamines and serotonin are not transported by this system, nor do they inhibit it [13]. Transport of these amines is facilitated by a carrier and is driven by a transmembrane electrochemical proton gradient.† In our previous studies, the coexistence of the amine transport system and beta-adrenergic receptors in the same cells required complex approaches to distinguish between receptor-mediated binding and the transport of beta-adrenergic antagonists such as propranolol and dihydroalprenolol (DHA‡).

In the present report we show that the rat pituitary tumor cell line GH_3 has an active transport system for DHA, although it does not have detectable beta-adrenergic receptors.

METHODS

Cells and culture conditions. Rat pituitary tumor cells (GH_3) were obtained from the American Type Culture Collection, Rockville, MD (ATCC, CCL821) and grown in Ham's F-10 medium containing glutamine (2 mM) plus NEAA and supplemented with 15% horse serum and 2.5% fetal calf serum. Rat astrocytoma cells (C6) were obtained from ATCC (CCL107) and grown in Dulbecco's minimum essential medium (DMEM) plus 5% fetal calf serum; C6 cells were received at passage 39 and were not used above passage 60. Both cell lines were incubated at 37° in a humidified, 5% CO_2 atmosphere.

Chemicals. (−)-[Ring, propyl-^{3}H(N)]dihydroalprenolol (specific radioactivity 95.7 to 105.2 Ci/mmole), cyclic AMP radioimmunoassay kits, and Aquasol were obtained from New England Nuclear, Boston, MA. (±)-[^{3}H]CGP-12177 (specific radioactivity 42.7 Ci/mmole) was obtained from the Amersham Corp., Arlington Heights, IL. 3-*N*-Morpholinopropane sulfonic acid (MOPS), free acid and Na^+ salt were from Research Organics Inc., Cleveland, OH. (−)-Propranolol and (+)-propranolol

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‡ Abbreviations: DHA, (−)-[ring, propyl-^{3}H(N)]dihydroalprenolol; MOPS, 3-*N*-morpholinopropane sulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; EGTA, ethyleneglycolbis (amino-ethylether)tetra-acetate; and NEAA, non-essential amino acids.

were gifts from Ayerst Laboratories, New York, NY. Forskolin was obtained from the Calbiochem-Behring Corp., La Jolla, CA. All other chemicals were from the Sigma Chemical Co., St. Louis, MO.

Binding and uptake assays. Binding of CGP-12177 and uptake of DHA were measured as previously described [13]. Assay buffer consisted of MOPS (25 mM) containing NaCl (125 mM) and, when whole cells were assayed, it also contained isobutylmethylxanthine (IBMX, 5 mM) at pH 8.2 unless otherwise noted. Cells were harvested by scraping, washed with 5 ml of assay buffer, and suspended in assay buffer; final cell concentration was 2×10^5 cells/tube in a final volume of 0.5 ml in glass tubes. Alternatively, cells were harvested by decanting the medium and incubating cells with assay buffer containing 10 mM EGTA for 5 min at 37°; this solution was removed and the plate tapped to dislodge cells. Cells were suspended in 10 ml of assay buffer without EGTA, centrifuged, and resuspended to final volume. Viability was greater than 90% as determined by trypan blue exclusion. 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 of less than 1% had no effect on DHA uptake in intact cells. Ethanol (0.95%) inhibited DHA uptake in membrane vesicles by 20%, and results are compared with the ethanol-treated control. Assays were performed in triplicate and initiated by the addition of the radioactive ligand. Uptake was defined as the total cell-associated ligand minus the filter background. Partially purified membrane vesicles were prepared by allowing cells to swell for 10 min on ice in 1 mM MOPS (pH 8.2) containing phenylmethylsulfonyl fluoride (0.5 mM) and then lysing the cells with 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 centrifuged at 30,000 *g* for 20 min, and the resulting pellet was suspended in assay buffer with the aid of a Brinkmann Polytron homogenizer set at speed 4 for 15 sec. About 100 μg of membrane protein was added to each assay tube, and assays were performed as described for whole cells.

Cyclic AMP production. Cyclic AMP production in response to beta-adrenergic agonists or forskolin was determined by suspending 4×10^5 cells in 0.5 ml assay buffer (pH 8.1) with or without agonists or forskolin for 15 min at 37°. Endogenous levels of cAMP were determined in cells incubated in an ice bath. After incubation, assay tubes were placed in a boiling water bath for 4 min and centrifuged, and cAMP in the supernatant fluid was measured by radioimmunoassay (RIA).

Miscellaneous. Protein was estimated by a modification [14] of the method of Lowry *et al.* [15] with bovine serum albumin as standard.

RESULTS

As shown in Table 1, cyclic AMP levels in the rat glioma cell line C6 were elevated markedly by the beta-adrenergic agonists isoproterenol, epinephrine, and norepinephrine. In contrast, cAMP levels in GH_3 cells were unaffected by these beta-agonists. No response was obtained in GH_3 when isoproterenol was tested over the concentration range of 1 nM to 0.1 mM (data not shown). Forskolin, which stimulates adenylate cyclase in a receptor-independent manner [16] was equally effective in elevating cAMP in both cell types, stimulating production 19- and 16-fold over basal levels in C6 and GH_3 respectively (Table 1). Therefore, although GH_3 cells have a functional adenylate cyclase, cAMP production is not stimulated by the 3 beta-adrenergic agonists tested.

Table 1. Stimulation of cAMP production in intact GH_3 and C6 cells

Addition	cAMP produced (pmoles/mg protein)	
	GH_3 cells	C6 cells
None	392	105
Isoproterenol (10 μM)	396	1935
Epinephrine (10 μM)	361	1644
Norepinephrine (10 μM)	365	1704
Forskolin (10 μM)	6218	1965

Rat pituitary tumor cells (GH_3) and rat glioma cells (C6) were grown, harvested, and assayed as described in Methods. Cells were incubated for 15 min at 37° in a final volume of 500 μl in MOPS (25 mM) + NaCl (125 mM) + IBMX (5 mM), pH 8.1. Cyclic AMP production was measured by RIA; results of a representative experiment in a series of four experiments giving qualitatively identical results are shown, with values representing total cAMP minus endogenous level.

As shown in Table 2, membranes prepared from GH_3 cells did not bind the partial agonist [^{3}H]CGP-12177 [17] in a manner suggesting the presence of beta-adrenergic receptors. The small amount of [^{3}H]CGP-12177 bound was not displaced by either (+)- or (−)-propranolol at either 10^{-4} M or 10^{-5} M. Comparable results were obtained in several experiments at higher and lower concentrations of [^{3}H]CGP-12177. For example, with the radioactive ligand present at 10 nM, 49 and 51 fmoles/mg protein were bound in the presence of 10^{-5} M and 10^{-4} M (−)-propranolol respectively. In many other experiments with intact GH_3 cells grown in both monolayer and suspension culture, comparable low levels of

Table 2. Binding of [^{3}H]CGP-12177 to GH_3 cell membranes

Competitive ligand	[^{3}H]CGP-12177 bound (fmoles/mg protein)
None	31.3 ± 4.0 (N = 6)
(+)-Propranolol, 10 μM	33.4 ± 5.8 (N = 5)
(−)-Propranolol, 10 μM	28.2 ± 1.5 (N = 4)
(+)-Propranolol, 100 μM	27.5 ± 2.5 (N = 6)
(−)-Propranolol, 100 μM	28.4 ± 3.9 (N = 5)

GH_3 cells were grown, membranes were prepared, and binding of [^{3}H]CGP-12177 (5 nM) was measured as described in Methods. Results are expressed as the mean of the indicated number (N) of samples ± the standard error of the mean at the 95% confidence level.

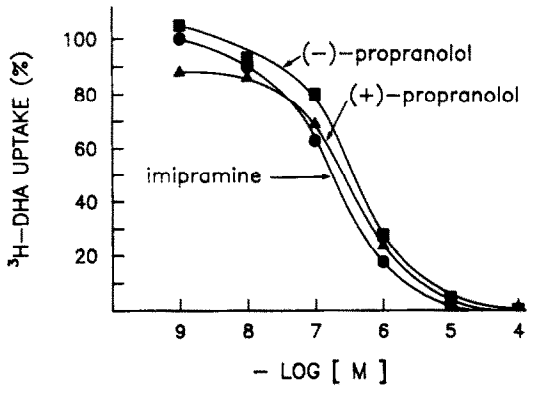

Fig. 1. Inhibition of DHA uptake in GH_3 cells by (−)- and (+)-propranolol and imipramine. GH_3 cells were incubated with radiolabeled DHA (2.5 nM) and increasing concentrations of the indicated competitor in assay buffer (pH 8.2, 20°) and filtered rapidly when equilibrium was reached (20 min). Results are expressed as percent of total cell-associated DHA in the absence of competitor. See Methods for details.

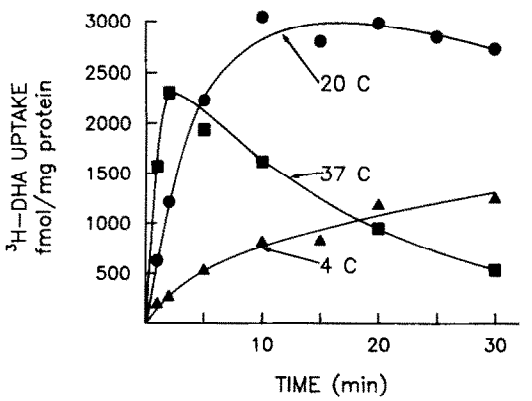

Fig. 3. Effects of temperature on the association of DHA with GH_3 cells. GH_3 cells were incubated in assay buffer (pH 8.2) containing 2.5 nM radiolabeled DHA at the indicated temperature and filtered rapidly at the indicated time. Results are expressed as the total cell-associated ligand minus the filter background. See Methods for details.

[^{3}H]CGP-12177 binding were found which again were not displaced by (+)- or (−)-propranolol (data not shown).

In contrast to CGP-12177, the beta-adrenergic antagonist DHA became associated with GH_3 cells to a high level; more than 12% of the total added ligand was taken up. Propranolol competition for the association of DHA with GH_3 cells was not stereoselective, as shown in Fig. 1. In addition, the tricyclic antidepressant imipramine was fully as effective as (+)- or (−)-propranolol in competing for DHA uptake (Fig. 1); each of these compounds had a 50% inhibitory concentration (IC_{50}) of about 0.3 μM (Fig. 1). Dichloroisoproterenol and alprenolol also competed effectively for DHA uptake (IC_{50}) = 1 μM); in contrast, isoproterenol, epinephrine, norepinephrine, serotonin and dopamine did not compete for DHA uptake in GH_3 cells (data not shown). Saturation of GH_3 cells with DHA was obtained at concentrations far greater than those typical of receptor-mediated binding; as shown in Fig. 2, saturation was obtained at a high DHA concentration, with an apparent K_m of 1.4 μM. Also, DHA uptake in GH_3 cells was sharply pH dependent; uptake at pH 6.2 and 7.2 was only 73 and 499 fmoles/mg protein respectively, compared to the 3109 fmoles/mg protein taken up at pH 8.2. Finally, as shown in Fig. 3, comparison of the association of DHA with GH_3 cells at 4°, 20° and 37° shows a marked temperature effect; this observation is consistent with the hypothesis that the association of DHA with GH_3 cells represents transport rather than diffusion [18]. In preliminary experiments, the rate of efflux of radiolabeled DHA from preloaded, intact GH_3 cells when diluted 10-fold in buffer was accelerated 3-fold by the inclusion of a 1000-fold excess of alprenolol in the buffer. Both inhibition by reserpine [19] and accelerative exchange diffusion [20] are evidence for the involvement of a carrier in ligand transport.

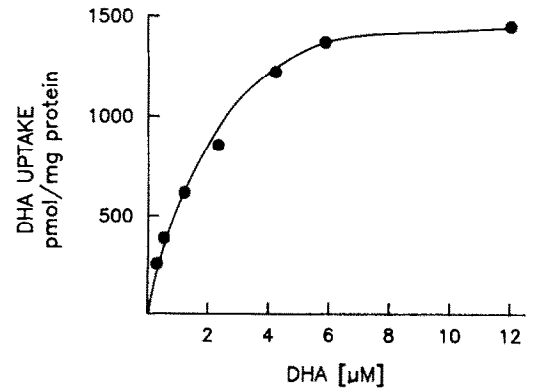

Fig. 2. Saturation of GH_3 cells with radiolabeled DHA. DHA was diluted to 0.16 Ci/mmole with unlabeled (−)alprenolol. GH_3 cells were incubated in assay buffer (pH 8.2, 20°) containing the indicated concentration of radiolabeled DHA until equilibrium was reached (20 min) and then filtered rapidly. See Methods for assay details. Results are expressed as total cell-associated DHA minus filter background. An apparent K_m was derived from a double-reciprocal plot using the line determined by regression analysis of these data.

We have shown previously that the transport of DHA into vesicle preparations from HeLa and C6 cells is dependent on the presence of MgATP to generate an electrochemical proton gradient as the driving force [13]. As shown in Table 3, this was also the case with GH_3 cells; transport of DHA into vesicles prepared from GH_3 cells was dependent on the addition of MgATP, which could not be replaced by Mg^{2+}, by ATP alone, or by CaATP or MgADP. DHA transport in GH_3 vesicles was inhibited by the ATPase inhibitor DCCD and by NEM. Reserpine, known to inhibit MgATP-dependent catecholamine and serotonin transport in chromaffin granules [19–22], synaptic vesicles [23, 24], and platelet storage organelles [25], was a potent inhibitor of DHA uptake in intact GH_3 cells and in membranes prepared from them (Table 4). The Na^+/H^+ ion-

Table 3. Nucleotide requirements and inhibitor effects on DHA uptake in membrane vesicles from GH_3 cells

MgATP present	Additives	DHA uptake* (%)
+	None	100
−	ATP (2.5 mM)	29
−	Mg^{2+} (2.5 mM)	2
−	CaATP (2.5 mM)	11
−	MgADP (2.5 mM)	7
+	DCCD (50 μM)	8
+	NEM (50 μM)	7

Vesicles were prepared from GH_3 cells as described in Methods and incubated in assay buffer (pH 8.2, 20°) with 2.5 nM radiolabeled DHA plus the indicated nucleotides or inhibitors. MgATP was present at 2.5 mM where indicated.

* Results are expressed as percent of total uptake in the presence of 2.5 mM MgATP minus the filter background; the 100% value for DHA uptake in this representative experiment was 168 fmoles/mg protein.

Table 4. Effects of inhibitors on [^{3}H]DHA uptake in intact cells and membrane vesicle preparations from GH_3 cells

	[^{3}H]DHA uptake (fmoles/mg protein)	
Inhibitors	Intact cells	Vesicles
None	3109	374
Reserpine, 10 μM	248	42
Monensin, 10 μM	121	14
Oligomycin*	171	411
Ouabain, 1 mM	2773	ND†

GH_3 cells were grown and harvested, vesicles were prepared, and assays were conducted as described in Methods. [^{3}H]DHA transport was measured at pH 8.2 in assay buffer containing 2.5 nM [^{3}H]DHA. Intact cells were incubated at 20° and 5 mM IBMX included; vesicles were incubated at 37° and 5 mM each of $MgCl_2$ and ATP included.

* Oligomycin was present at 118 μM with intact cells and 5 μg/mg protein with membrane preparations.

† ND = not determined.

ophore monensin, which dissipates proton gradients [26], was also a strong inhibitor of DHA uptake in GH_3 cells and membranes (Table 4). Oligomycin, which inhibits mitochondrial ATPases [27], also inhibited DHA uptake in intact GH_3 cells (Table 4). Oligomycin did not inhibit DHA transport in GH_3 membrane vesicles at 5 μg/mg membrane protein, a concentration shown to completely inhibit submitochondrial particle proton-pumping ATPases but not the clathrin-coated vesicle proton pump [28]. The Na^+/K^+ pump inhibitor, ouabain, at a concentration of 1 mM, only inhibited DHA transport 10% in GH_3 cells (Table 4). This pattern of nucleotide activities and oligomycin insensitivity is identical to the one described for the proton-pumping ATPase found in clathrin-coated vesicles [28, 29]

DISCUSSION

In this report we demonstrate that the rat pituitary tumor cell line GH_3 does not contain detectable beta-adrenergic receptors, but does contain an active transport system for the beta-adrenergic antagonist DHA. This transport system appears to be identical to the one we have recently described in several other cell types [13]. Therefore, we conclude that the amine transport system and the beta-adrenergic receptor are discreet, independent entities.

The first conclusion—that GH_3 lacks beta-receptors—is based on: (a) our failure to detect specific, stereoselective binding of the beta-adrenergic ligands CGP-12177 and DHA to GH_3 cells; and (b) our inability to demonstrate stimulation of cAMP production in intact GH_3 cells by several beta-receptor agonists despite our demonstration of a functional, forskolin-stimulated adenylate cyclase in GH_3. In the absence of hormonal stimulation, GH_3 cells appeared to synthesize about 3- to 4-fold more cAMP than C6 glioma cells during the 15-min incubation; this may be a reflection of a fundamental difference between the two cell lines or it may be a function of the different growth media used in each case. The increase in cAMP synthesis in response to forskolin stimulation was nearly the same for GH_3 (16-fold) and C6 (19-fold). These results indicate that GH_3 cells possess a functional adenylate cyclase; the existence of a functional, forskolin-stimulated adenylate cyclase in GH_3 cells has been described by others [30]. The inability of beta-adrenergic agonists to stimulate cAMP production in GH_3 could represent a defect in the regulatory proteins of the adenylate cyclase system but, together with the lack of specific binding of CGP-12177 or DHA, the evidence suggests an absence of receptors. Therefore, based on the two standard criteria for description of these receptors, i.e. ligand binding and adenylate cyclase stimulation by appropriate agonists, we conclude that GH_3 cells do not possess beta-adrenergic receptors. We are not aware of any reports that GH_3 cells do possess such receptors.

Our second conclusion—that GH_3 has an active transport system for basic amines—is based on the demonstration of DHA transport by a selective, saturable, high capacity, nonstereospecific, energy-requiring system. Uptake of the beta-adrenergic antagonist DHA by GH_3 was markedly pH and

temperature dependent and exceeded 12% of the ligand added. In contrast to receptor-mediated binding, which usually saturates with ligand in the nanomolar range, GH_3 cells only became saturated with DHA at a concentration of about 5 μM. Using approximations to calculate the intracellular volume of GH_3 cells, DHA appeared to be concentrated more than 300-fold within the cells compared to the extracellular concentration; we have previously shown DHA to concentrate about 340-fold in HeLa cells [13]. The question of sequestration of DHA in intracellular organelles was not addressed in these studies, but sequestration did not appear to be the case in our previous studies with HeLa cells in which we showed that reserpine pretreatment did not enhance ionophore-mediated ligand efflux from preloaded cells. Transport of DHA in GH_3 cells also meets two criteria for carrier-mediated transport, viz. inhibition by reserpine [19] and acceleration of exchange diffusion by unlabeled ligand [20]. It should also be noted that Vatner and Lefkowitz in 1974 [31] described a high-capacity, nonreceptor binding site for propranolol which appears to share some features with the transport site we describe in this report.

The system we describe recognizes DHA, propranolol, and imipramine but not catecholamines or serotonin; its selectivity, therefore, is clearly different from that of chromaffin granules [19, 22]. However, it appears to resemble previously described mechanisms for accumulation of propranolol, imipramine, and certain other basic amines in perfused lung [32, 33] and, in more recent reports, by isolated alveolar macrophages [34, 35]. In the latter reports, an active uptake mechanism has been suggested but has not been elucidated.

From our results we conclude that DHA is taken up by GH_3 cells via a carrier-mediated, energy-requiring transport process; transport appears to be driven by an ionophore-sensitive proton gradient which requires energy for its maintenance. In membrane vesicles prepared from these cells, nucleotide requirements and drug sensitivity patterns indicate that a nonmitochondrial proton-pumping ATPase identical to the "energy transformer" recently described by Racker and his colleagues in clathrin-coated vesicles [28, 29] is responsible for maintenance of the proton gradient.

As this manuscript neared completion, we became aware of a recent report describing the uptake of DHA by HeLa cells [36] in which the authors express the belief that DHA diffuses across the plasma membrane and accumulates in lysosomes. Staehelin *et al.* [17] had suggested previously that DHA uptake in C6 glioma cells was due to the lipophilic nature of the molecule. However, we have concluded that certain amines such as DHA are recognized by a specific carrier and transported by a motive force provided by a transmembrane electrochemical proton gradient. The demonstration of this transport system in a cell line lacking the complicating factor of coexisting beta-adrenergic receptors clearly establishes transport as a receptor-independent phenomenon, and should facilitate our progress toward understanding the system and its significance. The implications of a relatively high-affinity transport system for such widely-prescribed pharmacological agents as propranolol and imipramine are under investigation.

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