Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport

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Abstract U18666A and imipramine are hydrophobic amines that inhibit intracellular cholesterol transport pathways. In this study, we conducted dose-response curves for each of the cholesterol transport pathways. Our analyses indicate that hydrophobic amine inhibition of LDL-stimulated cholesterol esterification is much more sensitive to inhibition than either the combined bulk movement of cholesterol from lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum. Hydrophobic amines must inhibit a previously uncharacterized pathway from lysosomes to the endoplasmic reticulum or a signaling event that activates acyl CoA:cholesterol acyltransferase. Possible mechanisms for U18666A action were evaluated. The function of p-glycoprotein, which has been implicated in cholesterol transport, was unaffected by U18666A. We have evidence for a specific membrane U18666A binding site, which we hypothesize is involved in the plasma membrane to endoplasmic reticulum cholesterol transport pathway. Identification of the binding site and mechanism of hydrophobic amine action may provide information essential for understanding intracellular cholesterol transport.—**Underwood, K. W., B. Andemariam, G. L. McWilliams, and L. Liscum.** Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport. *J. Lipid Res.* 1996. **37:** 1556–1568.

Supplementary key words cholesterol • hydrophobic amine • U18666A • imipramine • transport • low density lipoprotein

Cholesterol is not uniformly distributed among cellular membranes but is localized primarily in the plasma membrane (1, 2). Cholesterol that is acquired by internalization and lysosomal hydrolysis of plasma lipoproteins, such as low density lipoprotein (LDL), or synthesized in the endoplasmic reticulum is rapidly transported to the plasma membrane (3–5) where it mixes with the plasma membrane pool of cholesterol (6). Possible mechanisms for intracellular cholesterol movement include aqueous diffusion or transport via vesicles, soluble carrier proteins, or lipids. These mechanisms may work separately or together to move cholesterol within the cell (7).

Our focus is on defining the mechanism of intracellular cholesterol transport and identifying the cellular factors involved. Genetic evidence for the existence of such cellular factors comes from the analysis of mammalian cells with specific impairments in the egress of LDL-cholesterol from lysosomes. These include fibroblasts from individuals with Niemann-Pick disease type C (8–11) and Chinese hamster ovary (CHO) cell mutants selected for defective cholesterol trafficking (12–14).

Pharmacological evidence for regulated cholesterol movement comes from studies with the hydrophobic amines, 3-β-[2-(diethylamino)-ethoxy]androst-5-en-17-one (U18666A) and imipramine. U18666A first came to our attention from the work of Panini, Sexton, and Rudney (15) who showed that the drug inhibits LDL-mediated suppression of 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Our studies indicated that U18666A also abolishes LDL-mediated stimulation of cholesterol esterification and suppression of LDL receptor activity in CHO cells, which we attributed to impaired movement of LDL-cholesterol out of the lysosome (16). In addition to inhibiting the lysosome-to-plasma membrane pathway, U18666A also inhibits cholesterol transport from the plasma membrane to the endoplasmic reticulum and mitochondria (17). A second hydrophobic amine, imipramine, inhibits LDL-cholesterol egress from lysosomes (18), but not movement from the plasma membrane to the endoplasmic reticulum (17).

Abbreviations: LDL, low density lipoprotein; CHO, Chinese hamster ovary; U18666A, 3-β-[2-(diethylamino)-ethoxy]-androst-5-en-17-one; [^{3}H]CL-LDL, LDL that is labeled with [^{3}H]cholesteryl linoleate; EBSS, Earle's balanced salt solution; 25-HC, 25-hydroxycholesterol; K_d, dissociation constant; IC_{50}, 50% inhibitory concentration; B_{max}, number of binding sites; ACAT, acyl-CoA:cholesterol acyltransferase; β-VLDL, β-migrating very low density lipoprotein; ALLN, N-acetylleucylleucylnorleucinal.

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In this study, we quantified hydrophobic amine inhibition of cholesterol movement and investigated possible mechanisms of action. Our analysis indicates that U18666A and imipramine inhibition of LDL-stimulated cholesterol esterification involves more than simply blocking the bulk movement of cholesterol from lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum. Instead, they must inhibit a previously uncharacterized pathway or a signaling event. While progesterone is postulated to affect cholesterol transport by inhibiting p-glycoprotein (19, 20), U18666A has no effect on p-glycoprotein function. A hydrophobic amine binding site is revealed with characteristics indicating its involvement in U18666A action.

MATERIALS AND METHODS

Materials

Sodium [^{3}H]borohydride (10 Ci/mmol), [9,10-^{3}H]oleic acid (10 Ci/mmol), [1,2,6,7-^{3}H(N)]cholesterol (51 Ci/mmol), [1,2,6,7-^{3}H(N)]cholesteryl linoleate (88 Ci/mmol), cholesteryl [1-^{14}C]oleate (57 mCi/mmol), and [^{3}H]imipramine (50.3 Ci/mmol) were obtained from New England Nuclear. [G-^{3}H]vinblastine sulfate (23 Ci/mmol) was purchased from Amersham. U18666A was purchased from Biomol and was also a generous gift from Dr. Richard Cenedella, Kirksville College of Osteopathic Medicine, Kirksville, MO. Imipramine, stearylamine, sphinganine, and sphingomyelinase were from Sigma, while all steroids were obtained from Steraloids. Cholesterol oxidase was purchased from Beckman and Calbiochem. Tissue culture reagents were from Life Technologies or Sigma. Other chemicals were from Sigma unless otherwise indicated.

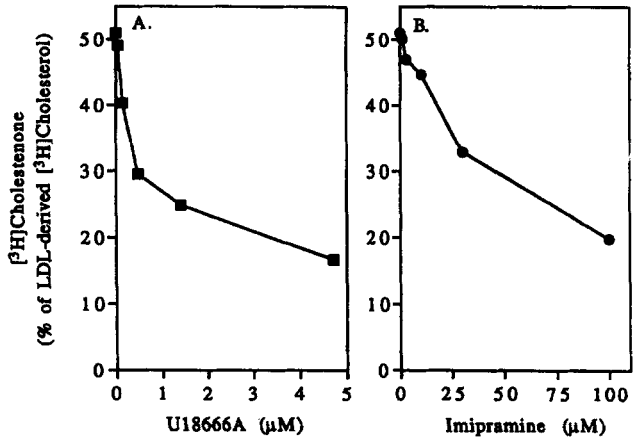

Fig. 1. Effect of U18666A and imipramine on movement of LDL-derived [^{3}H]cholesterol to the plasma membrane. Cells were grown and incubated with [^{3}H]CL-LDL in the absence and presence of U18666A (panel A) or imipramine (panel B), as described in Materials and Methods. Cells were treated with cholesterol oxidase after which the cellular contents of [^{3}H]cholesterol and [^{3}H]cholestenone were analyzed as described in Materials and Methods. The data points represent the percentage of cellular unesterified [^{3}H]cholesterol that was converted to [^{3}H]cholestenone, and represents the average of triplicate wells.

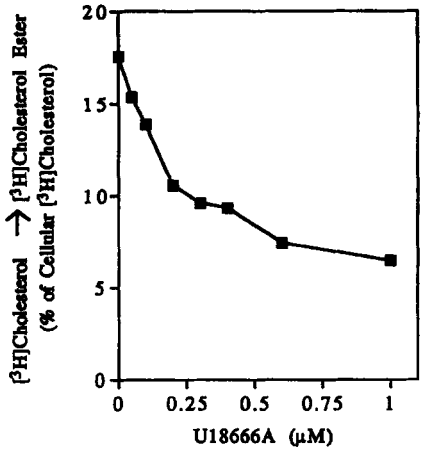

Fig. 2. Effect of U18666A on sphingomyelinase-stimulated [^{3}H]cholesterol esterification. Cells were grown and labeled with [^{3}H]cholesterol as described in Materials and Methods. Cells were treated with sphingomyelinase in the presence of the indicated concentration of U18666A, then cellular levels of [^{3}H]cholesteryl ester and [^{3}H]cholesterol were measured as described in Materials and Methods. The data points represent the percentage of cellular [^{3}H]cholesterol that was converted to [^{3}H]cholesteryl ester, and represents the average of duplicate wells.

Preparation of LDL, lipoprotein-deficient serum, media, and buffers

LDL was prepared by ultracentrifugation (21). [^{3}H-CL]-LDL was prepared with an average specific activity of 17,200 cpm/nmol of total cholesteryl linoleate (22). Lipoprotein-deficient serum was prepared from newborn calf serum, omitting the thrombin incubation (21). The following media were prepared: medium A [Ham's F-12 medium containing 5% (v/v) newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES, pH 7.1]; medium B [medium A in which 5% (v/v) newborn calf serum was replaced with 5% (v/v) lipoprotein-deficient calf serum]; medium C [medium B containing 20 μM mevinolin and 0.5 mM mevalonate]; and medium D [Ham's F-12 medium containing 20 μM mevinolin and 0.5 mM mevalonate]. The following buffers were prepared: TBS (50 mM Tris-Cl, pH 7.4, and 155 mM NaCl), TBS/BSA (50 mM Tris-Cl, pH 7.4, 155 mM NaCl, and 2 mg/ml bovine serum albumin), PBS (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl, at pH 7.3), and SEH (250 mM sucrose, 1 mM EDTA, and 20 mM HEPES, at pH 7.4).

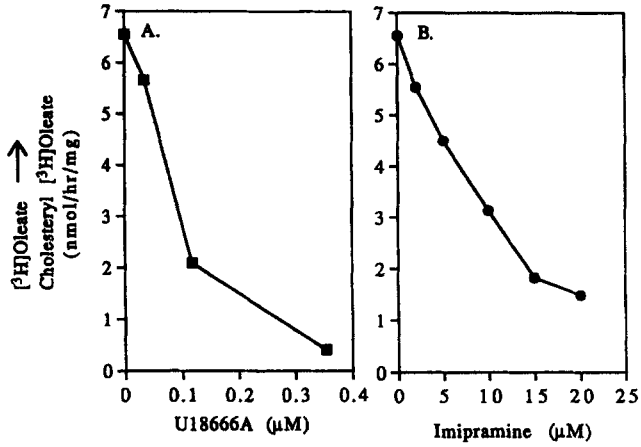

Fig. 3. Effect of U18666A and imipramine on LDL-stimulated cholesterol esterification. Cells were cultured as described in Materials and Methods. On day 3, cells were refed 1 ml/well medium C with 45 μg/ml LDL and the indicated concentrations of U18666A (■) or imipramine (●). After 8 h, cells were pulsed for 1 h with [^{3}H]oleate and the cellular concentration of cholesterol [^{3}H]oleate was determined as described in Materials and Methods. Each data point represents the average of duplicate wells.

Cultured cells and rat liver subcellular fractions

CHO-K1 cells were grown in monolayer in a humidified incubator (5% CO_2) at 37°C in medium A.

Livers were removed from adult Sprague-Dawley rats (>100 g) and homogenized in 4 volumes of ice-cold SEH with a Potter-Elvejham homogenizer. Nuclei and cellular debris were pelleted by centrifugation (2000 *g*, 10 min, 4°C). The postnuclear supernatant was subjected to centrifugation in a Ti70.1 rotor (Beckman Instruments) at 13,000 *g* for 15 min to obtain a crude lysosome/mitochondria pellet, and then at 100,000 *g* for 60 min to obtain a microsomal pellet and cytosolic supernatant.

Cholesterol oxidase treatment

On day 0, cells were seeded into 6-well plates (25,000 cells/well) in medium A. On day 1, cells were washed with Earle's balanced salt solution (EBSS) and refed 2 ml of medium B. Cells were refed 1 ml of medium B on day 3. On day 4, one set of cells was fed 1 ml of medium C, [^{3}H]CL-LDL, and the indicated concentrations of U18666A and imipramine. After an incubation period at 37°C, the cells were washed with TBS/BSA (3 times, 5 min each, 4°C), and PBS (3 times, 5 min each, room temperature). The cells were fixed (10 min, room temperature) with 1% (v/v) glutaraldehyde in PBS, and then washed in PBS (3 times, 2 min each, room temperature). The cells were incubated for 30 min at 37°C with 1 ml Ham's F-12 containing 0.1 unit of sphingomyelinase with or without 2 units of cholesterol oxidase (23). After washing with PBS (2 times, 2 min each, room temperature), lipids were extracted with hexane–isopropyl alcohol 3:2. A chromatography standard was added (20 μg cholestenone and 40 μg cholesterol), and [^{3}H]cholestenone and [^{3}H]cholesterol were separated by TLC using hexane–ethyl ether–glacial acetic acid 130:30:2 and visualized with iodine. Radioactivity was measured by liquid scintillation counting in ReadySafe (Beckman). [^{3}H]cholestenone formation is expressed as a percentage of total cellular [^{3}H]cholesterol and [^{3}H]cholestenone, and is the average of triplicate values. IC_{50} values were calculated by regression analysis of the data.

A separate set of cells was refed 1 ml of medium B containing 5 μCi [^{3}H]cholesterol on day 3. On day 4, the cells were refed 1 ml of medium C. After an incubation period at 37°C, the cells were fixed and cholesterol oxidase-treated exactly as described above.

Sphingomyelinase stimulation of cholesterol esterification

Cells were cultured using one of two formats. Format for the experiment shown in Fig. 2: on day 0, cells were seeded into 12-well plates (6,000 cells/well) in 2 ml of medium A. On day 1, cells were washed with EBSS and refed 1 ml of medium B. The cells were refed 1 ml of medium B containing 1 μCi [^{3}H]cholesterol on day 3, then refed 1 ml of medium B on day 4. Experiments were initiated on day 5. Format for Fig. 10: on day 0, cells were seeded into 6-well plates (25,000 cells/well) in 2 ml of medium A. On day 1, cells were washed with EBSS and refed 1 ml of medium B. The cells were refed

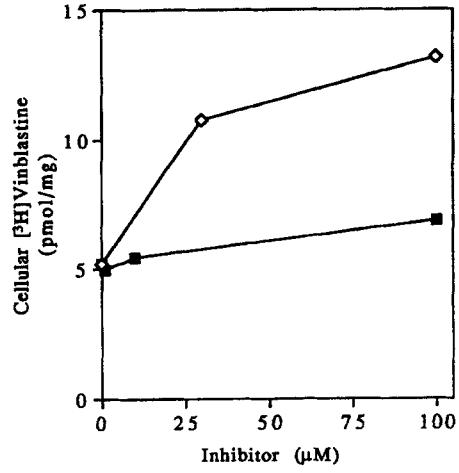

Fig. 4. Effect of U18666A and progesterone on the efflux of [^{3}H]vinblastine from CHO cells. Cells were grown as described in Materials and Methods. On day 2, cells were incubated with [^{3}H]vinblastine (50 nM, 0.1 μCi/ml) and the indicated concentrations of progesterone (◇) or U18666A (■). After 1 h, [^{3}H]vinblastine accumulation was determined as described in Materials and Methods and is expressed as pmol/mg of cellular protein. Each data point represents the average of triplicate wells.

1 ml of medium B containing 1 μCi [^{3}H]cholesterol on day 2. On day 3, the cells were refed 1 ml of medium B. After 1 h, cells were washed with EBSS and refed 1 ml of medium D containing sphingomyelinase (0.1 unit) (24) and the indicated concentration of U18666A and imipramine.

After 6 or 9 h at 37°C, the cells were washed with TBS (once quickly, once for 7 min, and once quickly, 4°C). Lipids were extracted with hexane–isopropyl alcohol 3:2 and a chromatography standard was added (20 μg cholesteryl oleate and 30 μg cholesterol). [^{3}H]cholesteryl oleate and [^{3}H]cholesterol were separated by TLC using toluene–ethyl acetate 2:1 and visualized with iodine. Radioactivity was measured by liquid scintillation counting in ReadySafe. [^{3}H]cholesteryl oleate formation is expressed as a percentage of total cellular [^{3}H]cholesterol, and represents the average of duplicate or triplicate samples. IC_{50} values were calculated by regression analysis of the data.

[^{3}H]vinblastine efflux

On day 0, cells were seeded into a 24-well plate (50,000 cells/well) in medium B. On day 2, cells were refed with 0.5 ml medium B containing 20 mM HEPES. After 1 h, the media were aspirated and the cells were refed 0.5 ml medium B containing [^{3}H]vinblastine (50 nM, 0.1 μCi/ml) and the indicated concentrations of progesterone and U18666A. The cells were incubated for 1 h after which time 10-μl aliquots were taken from two wells for liquid scintillation counting and measurement of specific activity of the [^{3}H]vinblastine. The cells were washed with Ham's F-12 containing 20 mM HEPES (3 times quickly, 4°C) and then solubilized in 0.1 N NaOH at room temperature for 30 min. Cell-associated radioactivity was measured by liquid scintillation counting of 0.425 ml solubilized cell extract in 15 ml Readysafe and 10 μl glacial acetic acid. Aliquots (50 μl) were also taken for protein determination using the method of Lowry et al. (25). The data are expressed as pmol of [^{3}H]vinblastine accumulation/mg cellular protein and represent the average of triplicate values.

Incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate

On day 0, cells were seeded into 6-well plates (25,000 cells/well) in 1.5 ml of medium A. On day 1, cells were refed 2 ml of medium B or medium C containing 3 μg/ml cholesterol. On day 3, cells were subjected to the incubations as indicated in the figure legends, then pulsed for 1 h with [^{3}H]oleate (16). After 1 h, cells were washed and [^{3}H]cholesteryl oleate was isolated (16). After the lipids were extracted, the monolayers were dissolved in 0.1 N NaOH and aliquots were taken for protein determination by the Lowry method (25). Cholesterol esterification is defined as nmol of cholesteryl [^{3}H]oleate formed per h/mg of protein, and represents

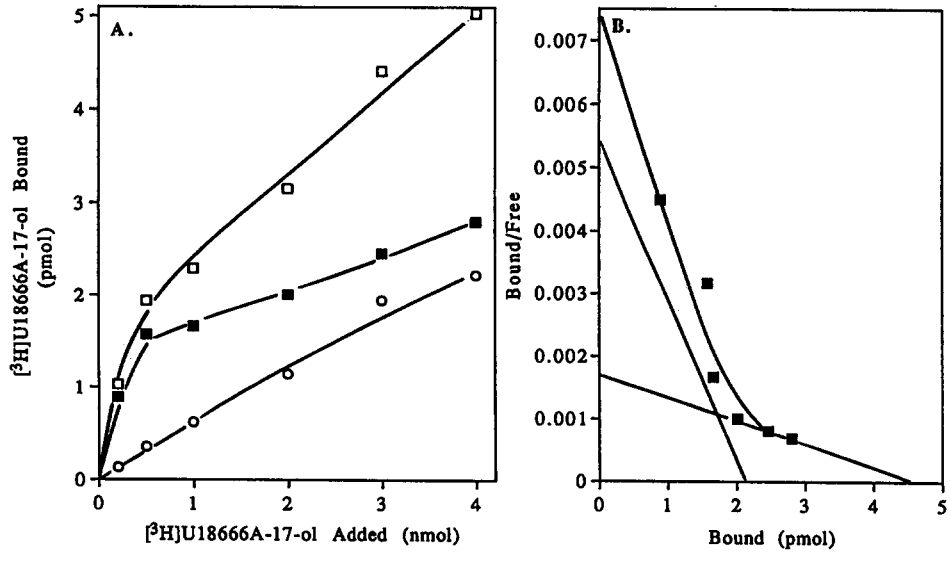

Fig. 5. Specific binding of [^{3}H]U18666A-17-ol to CHO cells. Cells were grown as described in Materials and Methods. Panel A: On day 4, cells were refed 0.5 ml medium B with (○) or without (□) 200 μM U18666A containing increasing concentrations of [^{3}H]U18666A-17-ol (500,000 cpm/nmol). After 60 min, cells were washed and cell-associated radioactivity was determined as described under Materials and Methods. Binding is expressed as pmol bound in the absence (□, total binding) or presence (○, nonspecific binding) of 200 μM U18666A. To determine specific binding (■), nonspecific was subtracted from total binding. Panel B: Scatchard plot of the binding data. Lines indicate linear regression of high and low affinity points.

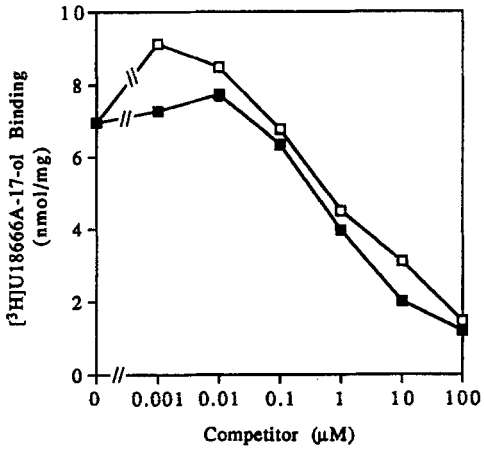

Fig. 6. U18666A (■) and U18666A-17-ol (□) inhibition of [^{3}H]U18666A-17-ol binding to CHO cells. Cells were grown as described in Materials and Methods. On day 3, cells received 0.5 ml medium B with the indicated concentration of U18666A (■) or U18666A-17-ol (□). After 15 min, [^{3}H]U18666A-17-ol (67 pmol) was added. After 45 min, cells were washed and cell-associated [^{3}H]U18666A-17-ol was determined as described in Materials and Methods. Binding is expressed as pmol/mg cellular protein.

the average of duplicate values. IC_{50} values were calculated by regression analysis of the data.

Preparation of [^{3}H]U18666A-17-ol

Preparation of the binding ligand [^{3}H]U18666A-17-ol required chemical reduction of the 17-ketone of U18666A to an alcohol with sodium [^{3}H]borohydride. In preliminary experiments, U18666A was reacted with 2 mole equivalents of sodium borohydride in ethanol for 30 min at 4°C. The reaction was quenched with water and products were extracted with petroleum ether. Thin-layer chromatography on silica gel G using a solvent system of $CHCl_3$–methanol–acetone–water–glacial acetic acid 97.5:20:5:2:3 separated unreacted U18666A (R_f 0.48) from a new species (R_f 0.36). The lower R_f indicated a more polar compound (i.e., containing a hydroxyl instead of a ketone). Mass spectrometry (kindly performed by Dr. Mark Nelson, Dept. Chemistry, Tufts University) revealed that the mass/charge ratio of U18666A was 388 and that borohydride reduction shifted the mass/charge ratio to 390. This is consistent with reduction of 3-β-[2-(diethylamino)ethoxy]-androst-5-en-17-one to 3-β-[2-(diethylamino)-ethoxy]androst-5-en-17-ol (referred to as U18666A-17-ol).

[^{3}H]U18666A-17-ol was prepared by reduction of 3 mg U18666A with 100 mCi sodium [^{3}H]borohydride in 100 μl 100% ethanol in an ice bath for 2 h. The reaction was quenched with an equal volume of water and products were extracted with petroleum ether. The extracted material was applied to a 4 × 40 mm Biosil A (Bio-Rad) column equilibrated with $CHCl_3$–methanol–acetone–water–glacial acetic acid 100:20:5:2:3 and eluted with the same solvent. Fractions containing [^{3}H]U18666A-17-ol were identified by thin-layer chromatography, pooled, and stored in 95% ethanol at -20°C. [^{3}H]U18666A-17-ol mass was measured by gas–liquid chromatography using a 3% OV-17 on 80/100 Gas Chrom Q column (6 feet by 0.085 inch, internal diameter; Alltech Associates, Deerfield, IL) and quantitated using a flame ionization detector with stigmasterol as an internal standard. The [^{3}H]U18666A-17-ol had a specific activity ranging from 0.7–7.1 × 10^6 cpm/μg (0.3–3.0 × 10^6 cpm/nmol).

Binding studies

On day 0, cells were seeded into 12-well plates (10,000–17,000 cells/well) in 1 ml of medium A. The cells were washed with EBSS and refed 1 ml medium B after 6 h or on day 1. The cells were refed 1 ml medium B on day 2 or 3. On day 4, cells were refed 0.5 ml medium B at 4°C. All subsequent steps were performed at 4°C. [^{3}H]U18666A-17-ol, [^{3}H]imipramine, and competitors (dissolved in 95% ethanol) were added as described in the legends. Wells not receiving competitors were given an equal volume of ethanol. After the indicated incubation, the cells were washed at 4°C with TBS/BSA (once quickly, and three washes at 2–4 min each) followed by TBS (once quickly, two washes at 2–4 min each, and once quickly). Cells were solubilized in 1 ml of 0.1 N NaOH at room temperature for 30 min. Cell-associated radioactivity was measured by liquid scintillation counting of 0.5 ml solubilized cell extract in 15 ml ReadySafe (Beckman) and 10 μl glacial acetic acid. Aliquots (0.2 ml) were also taken for protein determina-

TABLE 1. Inhibition of [3H]U18666A-17-ol binding by hydrophobic amines and U18666A analogues

Competitor	[3H]U18666A-17-ol Binding
	% of control
U18666A	34.2
Imipramine	33.9
Stearylamine	44.6
Sphinganine	27.1
5-Androsten-3β-ol-17-one	95.7
5-Androsten-3-17-dione	96.9
5-Androstan-3β-ol-17-one	103.7
5-Androsten-3β-17β-diol	103.8

Cells were grown as described in Materials and Methods. Cells were refed 1 ml of medium B on days 4 and 7. On day 8, cells were refed 0.5 ml of medium B with the indicated competitor at 100 μM. After 30 min, 1.6 nmol [3H]U18666A-17-ol was added. After 30 min, cells were washed and cell-associated [3H]U18666A-17-ol was determined as described under Materials and Methods. Binding is expressed as a percentage of the total binding in cells that received no competitor (528 pmol/mg).

tion using the Lowry method (25). The data are expressed as pmol of ligand bound/mg cellular protein, and represent the average of triplicate values.

Rat liver membranes were incubated with [^{3}H]U18666A-17-ol or [^{3}H]imipramine in PBS at 4°C. To separate bound from free ligand, the incubations mixes were filtered using GF/F glass fiber filters (Whatman) and a Millipore 1225 sampling manifold. Filters were washed three times with 10 ml TBS-BSA and subjected to scintillation counting in 10 ml of ReadySafe (Beckman). The data represent the average of triplicate values.

Rat liver cytosol (100,000 *g* supernatant) was incubated with [^{3}H]U18666A-17-ol at 4°C in 50 mM Tris-Cl, 100 mM KCl, 0.5 mM dithiothreitol at pH 7.3. To separate bound from free ligand, a 50% slurry of Bio-Rad HTP hydroxylapatite was added to the assay and incubated with agitation for 30 min at 4°C. The HTP was washed three times with TBS-BSA after which bound ligand was eluted with ethanol and subjected to liquid scintillation counting in ReadySafe.

TABLE 2. LDL and 25-hydroxycholesterol stimulation of cholesterol esterification in the absence and presence of U18666A and related analogues

Additions to Media	Cholesterol Esterification
	nmol/h/mg
Experiment 1	
None	0.9
LDL (30 μg/ml)	11.8
LDL plus 5-androsten-3β-ol-17-one (1 μM)	7.2
LDL plus 5-androsten-3-17-dione (1 μM)	10.9
LDL plus 5-androstan-3β-ol-17-one (1 μM)	11.5
LDL plus 5-androsten-3β-17β-diol (1 μM)	9.6
LDL plus U18666A (1 μM)	0.7
Experiment 2	
None	0.3
25-HC (1 μg/ml)	6.0
25-HC plus 5-androsten-3β-ol-17-one (1 μM)	5.1
25-HC plus 5-androsten-3-17-dione (1 μM)	5.1
25-HC plus 5-androstan-3β-ol-17-one (1 μM)	5.5
25-HC plus 5-androsten-3β-17β-diol (1 μM)	5.8
25-HC plus U18666A (1 μM)	6.1

Cells were grown as described in Materials and Methods. On day 3, cells were refed 1.5 ml of medium B. On day 4, cells were refed 1 ml of medium C with the indicated additions. After 6 h, monolayers were pulsed with [3H]oleate for 1 h. The cellular content of cholesteryl [3H]oleate was determined as described under Materials and Methods.

RESULTS

Sensitivity of cholesterol transport pathways to hydrophobic amine inhibition

U18666A is a hydrophobic amine that inhibits LDL-mediated regulatory responses, such as the stimulation of cholesterol esterification and suppression of endogenous cholesterol synthesis. The mechanism of U18666A action is unknown. One hypothesis is that U18666A blocks LDL stimulation of cholesterol esterification simply by blocking LDL-cholesterol transport pathways, for example, from lysosomes to the plasma membrane and then from the plasma membrane to the endoplasmic reticulum. To test this hypothesis, we quantified the sensitivity of several transport pathways to inhibition by U18666A.

Cholesterol transport from lysosomes to plasma membranes was tested by incubating cells for 2 h with [^{3}H]CL-LDL in the absence or presence of U18666A. The amount of LDL-derived [^{3}H]cholesterol that was sensitive to cholesterol oxidase treatment was determined using the method of Slotte et al. (23). The cholesterol oxidase-sensitive pool of cellular cholesterol is largely at the plasma membrane (26). In the absence of U18666A, 51% of the LDL-derived [^{3}H]cholesterol was converted to cholestenone. In other experiments, when the cells were incubated with [^{3}H]CL-LDL for 4 h, 84% of the LDL-[^{3}H]cholesterol was converted to [^{3}H]cholestenone. **Figure 1A** shows that U18666A caused a concentration-dependent sequestering of LDL-derived [^{3}H]cholesterol in a cholesterol oxidase-resistant pool, with an IC_{50} for U18666A action of 0.9 μM. The mean (± SD) IC_{50} for seven experiments was 0.8 μM (±0.3). A trivial explanation for this finding could be that U18666A directly inhibits cholesterol oxidase or alters the membrane to prevent accessibility of the enzyme to cholesterol; however, we found that plasma membrane [^{3}H]cholesterol was oxidized at normal levels when intact cells were incubated with U18666A at 100 μM (data not shown).

The pool of cholesterol oxidase-resistant LDL-derived [^{3}H]cholesterol is likely to be lysosomal in light of our earlier assessment of LDL-[^{3}H]cholesterol distribution using Percoll density gradients, which showed that U18666A at 2.3 μM inhibited the transfer of LDL-[^{3}H]cholesterol from lysosomes to other cellular membranes by 65% (9). In Fig. 1A, the movement of LDL-[^{3}H]cholesterol into the cholesterol oxidase-sensitive pool was inhibited by 65% with 2.5 μM U18666A, indicating that the two procedures for monitoring the distribution of LDL-[^{3}H]cholesterol gave equivalent results.

The cholesterol transport pathway from the lysosome to the plasma membrane was also tested for sensitivity to imipramine. Figure 1B shows that imipramine inhibited LDL-[^{3}H]cholesterol transport to a cholesterol oxi-

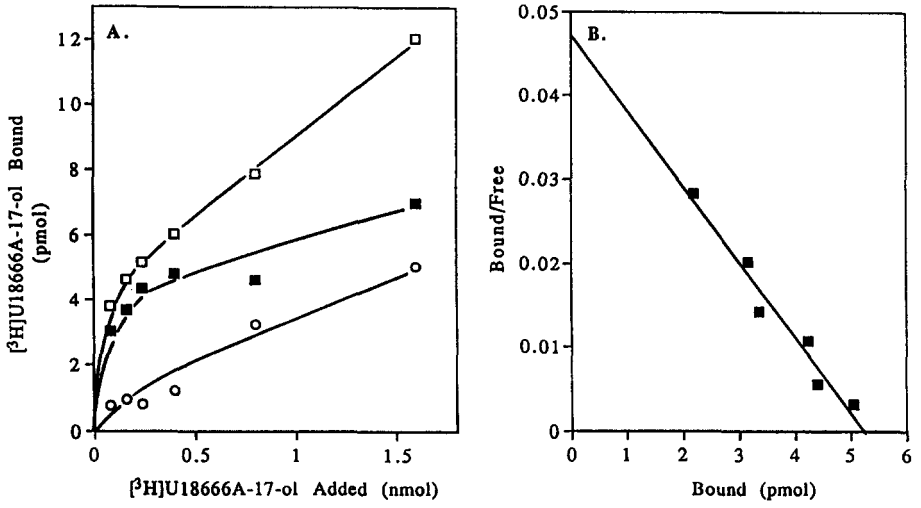

Fig. 7. Specific [^{3}H]U18666A-17-ol binding to rat liver microsomes. Panel A: Rat liver microsomes (100 μg) were incubated in PBS with (○) or without (□) 100 μM U18666A. After 30 min, increasing amounts of [^{3}H]U18666A-17-ol were added (311,000 cpm/nmol). After 30 min, membrane-bound radioactivity was determined as described under Materials and Methods. Binding is expressed as pmol bound in the absence (□, total binding) or presence (○, nonspecific binding) of 100 μM U18666A. To determine specific binding (■), nonspecific was subtracted from total binding. Panel B: Scatchard plot of the binding data. The line indicates linear regression analysis of the data.

dase-sensitive pool with an IC_{50} of 59 μM (mean ± SD for 3 experiments is 58 μM ± 10).

As a test of the cholesterol oxidase method, cells were also labeled with [^{3}H]cholesterol (dissolved in ethanol and added directly to the media) and the [^{3}H]cholesterol was allowed to equilibrate with cellular pools for 1 day. In six experiments, the mean (± SD) was 72% (±9) of cellular [^{3}H]cholesterol in a cholesterol oxidase-sensitive pool.

Cholesterol transport from the plasma membrane to the endoplasmic reticulum was analyzed using the sphingomyelinase method of Slotte and Bierman (24). CHO cells were cultured for 1 day with [^{3}H]cholesterol (added to the media dissolved in ethanol), then washed and cultured overnight to allow the [^{3}H]cholesterol to distribute with the cellular cholesterol pools. Cells were then treated for 9 h with sphingomyelinase in the absence or presence of U18666A, after which cellular levels of [^{3}H]cholesterol and [^{3}H]cholesteryl esters were determined. Without sphingomyelinase treatment, 4.4% of cellular [^{3}H]cholesterol was incorporated into [^{3}H]cholesteryl esters; sphingomyelinase treatment stimulated cholesterol esterification such that 17.6% of cellular [^{3}H]cholesterol was esterified (**Fig. 2**). As reported by Harmala et al. (17), U18666A caused a concentration-dependent inhibition of this stimulation. In this experiment, the IC_{50} for U18666A action was 0.5 μM; in six experiments, the mean (± SD) was 0.5 μM (±0.1). The effect of U18666A was not due to inhibition of sphingomyelinase, as U18666A at concentrations up to 2.8 μM had no effect on sphingomyelinase activity (data not shown; also ref. 17).

While the plasma membrane to endoplasmic reticulum pathway is inhibited by U18666A, Harmala et al. (17) found that imipramine up to 100 μM had no effect on sphingomyelinase-stimulated movement of plasma membrane cholesterol to the endoplasmic reticulum. Our results confirmed that finding (data not shown).

To rule out the possibility that U18666A directly inhibits acyl-CoA:cholesterol acyltransferase (ACAT), we tested 25-hydroxycholesterol stimulation of ACAT, in the presence of U18666A. U18666A does not inhibit 25-hydroxycholesterol stimulation of [^{3}H]oleate incorporation into cholesteryl [^{3}H]oleate at concentrations up to 4.7 μM (data not shown), and is not likely to inhibit ACAT directly. This is in agreement with Harmala et al. (17). We found that U18666A starts to inhibit 25-hydroxycholesterol-stimulated incorporation of [^{3}H]cholesterol into [^{3}H]cholesteryl ester at 50 μM (data not shown). At such high concentrations of U18666A, this inhibition may be due to nonspecific effects.

The above results show that cholesterol transport from lysosomes to plasma membrane and from plasma membrane to endoplasmic reticulum are both inhibited by U18666A with IC_{50}s in the 0.5 to 0.8 μM range. By contrast, LDL stimulation of cholesterol esterification is much more sensitive to U18666A inhibition. **Figure 3** shows an experiment in which cells were incubated for 7 h with 45 μg/ml LDL, which stimulated cholesterol esterification to 6.56 nmol/h per mg from basal levels

of 0.38 nmol/h per mg. The stimulation of esterification was inhibited by the inclusion of U18666A, with an IC_{50} of 0.08 μM. Eleven experiments yielded a mean (± SD) IC_{50} of 0.11 μM (±0.07). This result was surprising because the lysosome to plasma membrane and plasma membrane to endoplasmic reticulum transport pathways are not significantly inhibited at 0.11 μM U18666A.

Similarly, LDL stimulation of cholesterol esterification is much more sensitive to imipramine inhibition than is LDL-cholesterol movement to the plasma membrane. Imipramine inhibits LDL stimulation of cholesterol esterification with an IC_{50} of 8.0 μM (mean ± SD for 3 experiments is 7.3 μM ± 3.0); however, 8.0 μM imipramine inhibits LDL-cholesterol transport from lysosomes to the plasma membrane by only 10%. As cited above, cholesterol movement from the plasma membrane to the endoplasmic reticulum is not inhibited by imipramine at concentrations up to 100 μM (17).

Thus, LDL stimulation of cholesterol esterification is inhibited by concentrations of U18666A and imipramine that have minimal effects on cholesterol transport pathways. This suggests that LDL-cholesterol stimulation of ACAT involves more than the bulk movement of LDL-cholesterol from lysosomes to the plasma membrane and from there to the endoplasmic reticulum. It could indicate that a regulatory step or an additional pathway is involved that is more sensitive to hydrophobic amine inhibition.

Effect of U18666A on p-glycoprotein function

Many amphiphiles that inhibit intracellular cholesterol transport also block the action of p-glycoprotein, a plasma membrane transport protein responsible for the multidrug-resistant phenotype. It has been suggested that p-glycoprotein may play a role in normal cholesterol trafficking (19, 20). To test whether U18666A inhibits cholesterol transport by affecting p-glycoprotein function, cells were incubated with [^{3}H]vinblastine in the absence and presence of various concentrations of progesterone and U18666A (**Fig. 4**). After 1 h, the cellular content of [^{3}H]vinblastine was measured. Vinblastine is a substrate for p-glycoprotein and is readily effluxed from the cell. Progesterone inhibits the efflux and there is a concentration-dependent increase in cellular levels of [^{3}H]vinblastine. U18666A, however, had no significant effect on [^{3}H]vinblastine efflux at concentrations up to 100 μM.

Binding studies with hydrophobic amines

Our hypothesis is that hydrophobic amines bind to cellular proteins that modulate intracellular transport, or perhaps are directly involved in cholesterol movement. To probe for a U18666A binding site, we radiolabeled the drug by sodium [^{3}H]borohydride reduction of the 17-ketone to an alcohol. The reduced compound, U18666A-17-ol, exhibited the ability to inhibit LDL stimulation of cholesterol esterification, although it took 3.3-fold more U18666A-17-ol to achieve inhibition compared to the parent compound (data not shown).

We found that CHO cells have specific binding sites for [^{3}H]U18666A-17-ol. The binding of [^{3}H]U18666A-17-ol (67 pmol) to intact CHO cells reached steady-state within 15 min and was completely abolished by the presence of 100 μM unlabeled U18666A (data not shown). U18666A binding sites were determined by incubating intact CHO cells with increasing concentrations of [^{3}H]U18666A-17-ol (**Fig. 5A**). Scatchard analysis (Fig. 5B) of these data indicated the presence of two sites. The higher affinity site had a K_d of 764 nM and a B_{max} of 76.2 pmol/mg, or approximately 6.16×10^6 molecules per cell. The high affinity sites were seen consistently, with a mean (± SD, 5 experiments) B_{max} of 72 pmol/mg (±27.2) and K_d of 985 nM (±191). We were encouraged by the finding that the concentration of [^{3}H]U18666A-17-ol required for half-maximal occupancy of the binding site was within the range of inhibitory action on cholesterol transport from lysosomes to plasma membrane and from plasma membrane to endoplasmic reticulum. This experiment also showed an equal number of low affinity sites (5.11 μM, 91 pmol/mg); however, the number and K_d of the low affinity sites varied between experiments.

The relative ability of U18666A and U18666A-17-ol to bind to intact CHO cells was determined by measuring

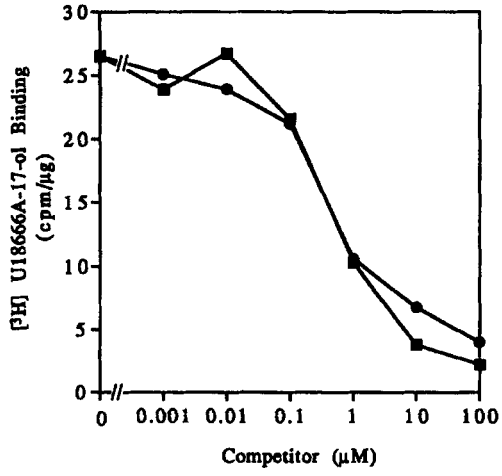

Fig. 8. Inhibition of [^{3}H]U18666A-17-ol binding in CHO cells by U18666A (■) and imipramine (●). Cells were grown as described in Materials and Methods. On day 4, cells received 0.5 ml medium B with the indicated concentrations of U18666A (■) or imipramine (●). After 15 min, [^{3}H]U18666A-17-ol (67 pmol) was added. After 30 min, cells were washed and cell associated [^{3}H]U18666A-17-ol was determined as described in Materials and Methods. Binding is expressed as pmol/mg cellular protein.

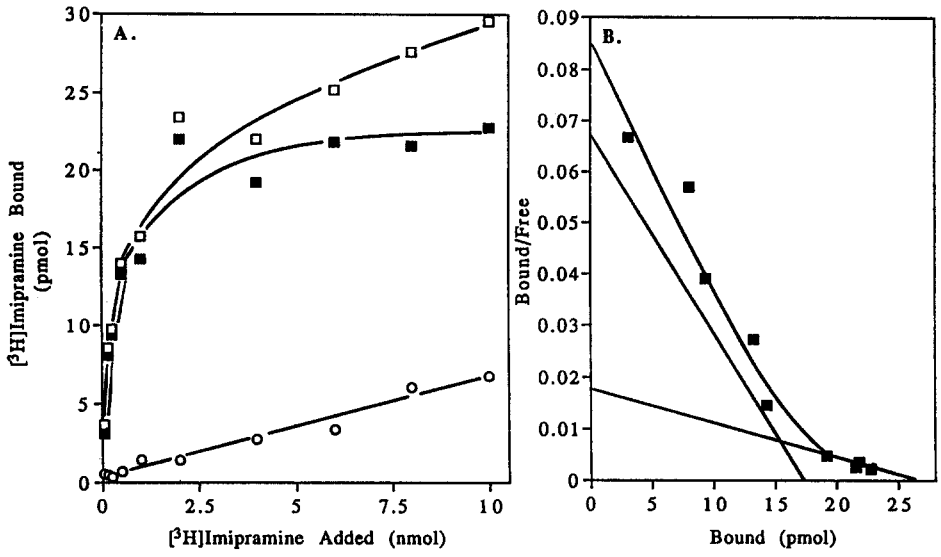

Fig. 9. Specific binding of [^{3}H]imipramine to CHO cells. Panel A: Cells were grown as described in Materials and Methods. On day 4, cells were refed 0.5 ml medium B with (○) or without (□) 200 μM imipramine containing increasing concentrations of [^{3}H]imipramine (5.6×10^7 cpm/nmol). After 30 min, cells were washed and cell-associated radioactivity was determined as described in Materials and Methods. Binding is expressed as pmol bound in the absence (□, total binding) or presence (○, nonspecific binding) of 200 μM imipramine. To determine specific binding (■), nonspecific was subtracted from total binding. Panel B: Scatchard plot of the binding data. The line indicates linear regression analysis of the data.

steady-state binding of 67 pmol [^{3}H]U18666A-17-ol in the presence of various concentrations of unlabeled U18666A and U18666A-17-ol. [^{3}H]U18666A-17-ol binding was reduced to 50% by inclusion of 0.95 μM U18666A (**Fig. 6**). Unlabeled U18666A-17-ol was 3-fold less effective than U18666A in inhibiting [^{3}H]U18666A-17-ol binding to intact CHO cells, with an IC_{50} of 2.8 μM, which is consistent with its being 3.3-fold less effective in inhibiting LDL responses such as cholesterol esterification. These data are also consistent with a physiologically relevant site being probed with [^{3}H]U18666A-17-ol.

Additional hydrophobic amines that inhibit LDL stimulation of cholesterol esterification and cause lysosomal accumulation of cholesterol include imipramine, stearylamine, and sphinganine (27). **Table 1** shows that these compounds all inhibited [^{3}H]U18666A-17-ol binding to CHO cells. We also tested 5-androsten-3β-17-one, which has the steroid ring structure of U18666A, as well as three related steroids. The four steroids had no effect on either [^{3}H]U18666A-17-ol binding (Table 1) or LDL or 25-hydroxycholesterol stimulation of cholesterol esterification (Table 2). NH_4Cl, at 20 mM, also had no effect on [^{3}H]U18666A-17-ol binding to intact CHO cells (data not shown), which indicates that the reduced [^{3}H]U18666A-17-ol binding by excess weak basic amines is not simply due to neutralization of acidic compartments in the cell.

The cellular distribution of [^{3}H]U18666A-17-ol binding sites was addressed by fractionating rat liver homogenate into lysosomes and mitochondria (13,000 *g* pellet), microsomes (100,000 *g* pellet), and cytosol (100,000 *g* supernatant). Specific [^{3}H]U18666A-17-ol binding to rat liver microsomes is shown in **Fig. 7A.** Scatchard analysis of the equilibrium binding data indicated a single class of sites with a B_{max} of 53 pmol/mg and a K_d of 222 nM (Fig. 7B). The lysosome/mitochondria fraction displayed similar sites (B_{max} of 33 pmol/mg and a K_d of 207 nM), while cytosol had fewer sites which were of low affinity (B_{max} of 7 pmol/mg and a K_d of 2 μM) (data not shown). Binding to microsomes and lysosome/mitochondria was not affected when microsomes were washed with 0.1 M sodium carbonate, which removes peripheral and lumenal proteins and flattens the microsomes (28).

The above data are all consistent with a membrane binding site involved in U18666A inhibition of cholesterol transport. However, one finding was inconsistent with this view. **Figure 8** shows the ability of imipramine to compete with [^{3}H]U18666A-17-ol for binding to intact CHO cells. We found that U18666A and imipramine inhibited [^{3}H]U18666A-17-ol binding to CHO cells equivalently, in this experiment with IC_{50}s of 0.7 and 0.6 μM, respectively. **Figure 9A** shows specific binding of [^{3}H]imipramine to CHO cells. Scatchard analysis

(Fig. 9B) of the equilibrium binding data revealed a high affinity site with a K_d of 516 nM and a B_{max} of 339 pmol/mg and a lower affinity site with a K_d of 2.91 μM and a B_{max} of 175 pmol/mg. These data are consistent with the number and affinity of [^{3}H]U18666A-17-ol binding sites; however, imipramine blocks LDL-cholesterol transport at 58 μM not 0.5 μM. Therefore, imipramine is binding to this site at a concentration that has no effect on cholesterol transport.

Competition studies in the plasma membrane to endoplasmic reticulum pathway

The hydrophobic amine binding site has many of the characteristics of a physiologically relevant site but we were puzzled by the finding that imipramine binds to the site itself and inhibits U18666A binding to the site at a low concentration. Our hypothesis is that this site mediates U18666A action on the plasma membrane to endoplasmic reticulum pathway. For this hypothesis to be correct, imipramine should interfere with U18666A's ability to block plasma membrane cholesterol transport and esterification. We tested this by evaluating U18666A inhibition of sphingomyelinase-stimulated movement of cholesterol from the plasma membrane to the endoplasmic reticulum in the absence and presence of 1 μM and 100 μM imipramine. Imipramine does not inhibit this pathway itself; however, if it interferes with U18666A inhibition of this pathway, then the IC_{50} for U18666A inhibition should increase, as it would take more U18666A to dislodge imipramine from the binding site and exert inhibitory action.

Figure 10A shows that the IC_{50} for U18666A action shifted 1.4-fold, from 0.65 μM to 0.90 μM in the presence of 1 μM imipramine. Figure 10B shows that the IC_{50} for U18666A action shifted 2.2-fold, from 0.26 μM to 0.58 μM in the presence of 100 μM imipramine. Therefore, in the presence of imipramine, it takes more U18666A to inhibit the plasma membrane-to-endoplasmic reticulum pathway despite the fact that imipramine itself has no inhibitory action. These data are consistent with our binding studies showing that imipramine competes with U18666A for binding to a membrane site and suggests that U18666A's inhibition of the plasma membrane to endoplasmic reticulum pathway is mediated by the drug binding site.

DISCUSSION

U18666A and imipramine belong to a class of compounds called hydrophobic amines, which inhibit intracellular cholesterol transport and LDL-mediated regulation of cellular cholesterol homeostasis (16–18, 29, 30). While U18666A and imipramine have been studied most extensively, the list of similarly acting compounds that also cause lysosomal accumulation of cholesterol includes the hydrophobic amines sphinganine, stearylamine, and RV 538 (27) and the steroid, progesterone (31). The mechanism of action of these compounds is

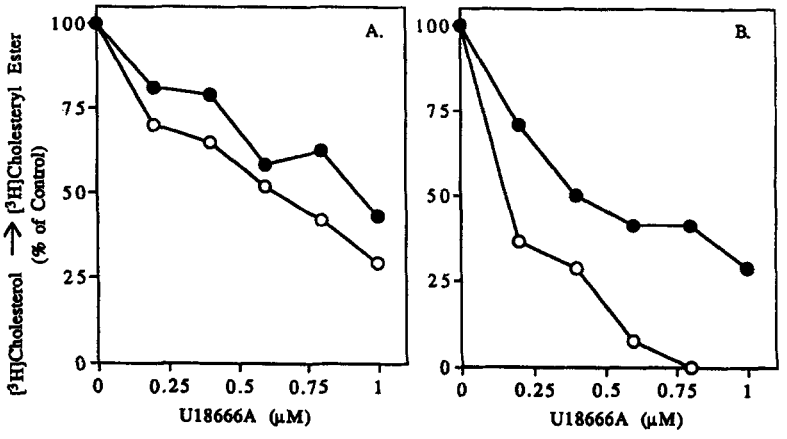

Fig. 10. Effect of imipramine on U18666A inhibition of sphingomyelinase-stimulated [^{3}H]cholesterol esterification. Cells were grown and labeled with [^{3}H]cholesterol as described in Materials and Methods. Cells were treated with sphingomyelinase in the presence of the indicated concentration of U18666A with (●) and without (○) 1 μM (panel A) or 100 μM (panel B) imipramine. Cellular levels of [^{3}H]cholesteryl ester and [^{3}H]cholesterol were measured as described in Materials and Methods, and the percentage of cellular [^{3}H]cholesterol that was converted to [^{3}H]cholesteryl ester was calculated. The data are expressed as a percentage of the control incubations that received no U18666A; these control values were 6.4 and 8.5% without and with imipramine, respectively. The data represent the average of triplicate wells.

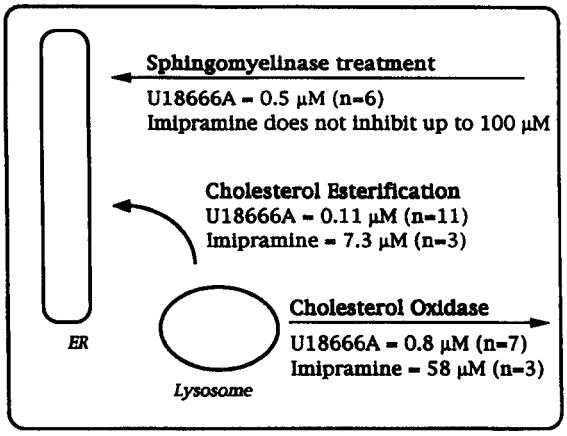

Fig. 11. Sensitivity of cholesterol transport pathways to inhibition by U18666A and imipramine. IC_{50} values are shown for the action of U18666A and imipramine on LDL-cholesterol movement from lysosomes to the plasma membrane, on the movement of plasma membrane cholesterol to ACAT in the endoplasmic reticulum, and on LDL stimulation of cholesterol esterification.

unknown, although progesterone is postulated to act through inhibition of p-glycoprotein (32).

We originally hypothesized that hydrophobic amines inhibit LDL-mediated regulatory events, such as stimulation of cholesterol esterification and suppression of cholesterol synthesis, due to their inhibition of LDL-cholesterol movement out of lysosomes. In this study, we tested that hypothesis by conducting dose-response curves for each of the cholesterol transport pathways inhibited by hydrophobic amines. Possible mechanisms for hydrophobic amine action were also evaluated. We determined the following.

U18666A blocks bulk cholesterol movement from lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum; however, these actions cannot account for the exquisitely sensitive U18666A inhibition of LDL stimulation of cholesterol esterification. **Figure 11** shows IC_{50}s for each pathway, which were determined by dose-response curves. At 0.1 μM U18666A, LDL-derived cholesterol movement from lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum is inhibited only 10%. Similarly, at 8 μM imipramine, movement of LDL-derived cholesterol from lysosomes to the plasma membrane is inhibited only 10% and movement from the plasma membrane to the endoplasmic reticulum is unaffected. Yet, in both cases, ACAT is not affected by this expansion of cellular cholesterol levels. This could indicate that cholesterol transit from the lysosomes to ACAT in the endoplasmic reticulum involves an additional pathway that is more sensitive to hydrophobic amine inhibition. For example, this might be a direct pathway from lysosomes to the endoplasmic reticulum or through the Golgi apparatus.

Alternatively, hydrophobic amines may inhibit a regulatory event that signals to ACAT that cellular cholesterol pools are expanded. This idea is consistent with the results of Schissel et al. (33), who found that ALLN inhibits a proteolytic step that is necessary for ACAT activation after the expansion of cellular cholesterol pools. They incubated macrophages and CHO cells with β-VLDL in the presence of ALLN and found normal β-VLDL degradation and lipoprotein-cholesterol transport out of lysosomes but no cholesterol esterification. Stimulation of cholesterol esterification by agents that do not expand cellular pools of cholesterol (i.e., 25-hydroxycholesterol and sphingomyelinase) were unaffected by ALLN. Similarly, low concentrations of U18666A and imipramine block LDL stimulation of cholesterol esterification while they do not block cholesterol transport out of lysosomes or stimulation of cholesterol esterification by 25-hydroxycholesterol or sphingomyelinase. ALLN does not act as a general inhibitor of cholesterol trafficking to ACAT, while U18666A and imipramine do inhibit cholesterol movement at higher concentrations.

U18666A does not affect the activity of p-glycoprotein, as measured by [^{3}H]vinblastine efflux, unlike the cholesterol transport inhibitor progesterone. It has previously been shown that U18666A does not alter the kinetics of cholesterol desorption from cellular membrane fractions or from intact CHO cell plasma membranes (16). However, it still remains possible that hydrophobic amines act by altering the lateral organization of membrane lipids.

U18666A binds to a membrane site with binding characteristics consistent with the site being involved in U18666A inhibition of cholesterol transport. The higher affinity site has a K_d of 985 nM for [^{3}H]U18666A-17-ol, which is in the concentration range of U18666A's inhibitory action on cholesterol transport from lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum. While this seems to be rather low affinity, the true dissociation constant of these hydrophobic compounds is not calculable because the partition coefficient of the ligands between the aqueous and membrane phases has not been measured. Also, we do not know if the ligand binding site faces the aqueous or lipid milieu (34). U18666A-17-ol is 3-fold less effective than U18666A in inhibiting LDL stimulation of cholesterol esterification and 3.3-fold less effective as a competitor of [^{3}H]U18666A-17-ol binding.

The physiological relevance of binding is underscored by the finding that [^{3}H]U18666A-17-ol binding is competed by other hydrophobic amines that inhibit LDL cholesterol transport (imipramine, stearylamine, sphinganine) but not by steroids that are structurally similar to U18666A, yet have no inhibitory activity. U18666A

and imipramine appear to be binding to the same site as excess imipramine blocks [^{3}H]U18666A-17-ol binding and excess U18666A blocks [^{3}H]imipramine binding.

The U18666A binding site has the characteristics of an integral membrane component. Subcellular fractionation of rat liver homogenate revealed the high affinity site on lysosome/mitochondrial and microsomal membranes, but not on cytosolic proteins. High affinity binding to rat liver microsomes was not affected by sodium carbonate treatment, which produces flattened membranes devoid of peripheral proteins. This also suggests that we were measuring binding and not uptake of ligand into organelles.

The above information is all consistent with the binding site being involved in cholesterol egress from lysosomes. However, one piece of information that does not agree with this model is the K_d for [^{3}H]imipramine binding of 516 nM. Imipramine does not have inhibitory activity at that concentration. Our hypothesis is that the binding site is involved in the plasma membrane to endoplasmic reticulum cholesterol transport pathway. We have found that imipramine interferes with U18666A action on that pathway but does not exert inhibitory activity of its own. Identification of the hydrophobic amine binding site may reveal a protein critical for this cholesterol transport pathway.

Our findings are consistent with U18666A inhibiting both bulk transport pathways and signaling to ACAT that cellular cholesterol pools are expanded. The work of Harmala and colleagues (17) suggests an additional action of U18666A. Sphingomyelinase digestion of plasma membrane sphingomyelin stimulates the rapid redistribution of up to 50% of plasma membrane cholesterol into a cholesterol oxidase-resistant pool (17), although only 5–10% of the cholesterol is found as cholesteryl ester. U18666A blocks both the sphingomyelinase-induced cholesterol esterification and the redistribution of plasma membrane cholesterol into a cholesterol oxidase-resistant pool (17). Recently, Porn and Slotte (35) have found that while some plasma membrane cholesterol does redistribute to cell interior upon sphingomyelinase treatment, most of the cholesterol stays at the plasma membrane. There it appears to associate tightly with phospholipids other than sphingomyelin. Given that U18666A blocks the movement of plasma membrane cholesterol into a cholesterol oxidase-resistant pool, it must act by preventing this lateral reorganization in addition to inhibiting sterol transport to the endoplasmic reticulum.

Strong candidates for gene products involved in cholesterol transport are lacking. Analysis of somatic cell mutants defective in cholesterol movement will provide substantial information on this process. In addition, elucidation of the mechanism of hydrophobic amine action will be valuable, as the hydrophobic amine binding site may be a cholesterol transport molecule, or reveal information on the transport mechanism. Alternatively, it may be a protein that indirectly modulates the organization of cholesterol in membranes.

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