We tested the hypothesis that certain membrane-intercalating agents increase the chemical activity of cholesterol by displacing it from its low activity association with phospholipids. Octanol, 1,2-dioctanoyl-sn-glycerol (a diglyceride), and N-hexanoyl-D-erythro-sphingosine (a ceramide) were shown to increase both the rate of transfer and the extent of equilibrium partition of human red blood cell cholesterol to methyl-β-cyclodextrin. These agents also promoted the interaction of the sterol with two cholesterol-specific probes, cholesterol oxidase and saponin. Expanding the pool of bilayer phospholipids with lysophosphatidies countered these effects. The three intercalators also protected the red cells against lysis by cholesterol depletion as if substituting for the extracted sterol. As is the case for excess plasma membrane cholesterol, treating human fibroblasts with octanol, diglyceride, or ceramide stimulated the rapid inactivation of their hydroxymethylglutaryl-CoA reductase, presumably through an increase in the pool of endoplasmic reticulum cholesterol. These data supported the stated hypothesis and point to competition between cholesterol and endogenous and exogenous intercalators for association with membrane phospholipids. We also describe simple screens using red cells in a microtiter well format to identify intercalating agents that increase or decrease the activity of membrane cholesterol.

Sterols associate in roughly equimolar proportions with the phospholipids in artificial membranes (1, 2). The sterols associated with the phospholipids are held at a low chemical activity. In sharp contrast, sterol molecules in excess of the capacity of the phospholipids are free and have a much higher chemical activity (3, 4). The high fugacity (escape potential) of the superthreshold sterol promotes its exit from the membrane, thereby tending to restore the balance between the sterols and phospholipids (2).

Most of the cholesterol in mammalian cells is in the plasma membranes, where its abundance appears to be maintained homeostatically near molar equivalence with the phospholipids (5, 6). Furthermore, cholesterol in excess of the physiological set point appears to have a high chemical activity. One indicator of this activity is the sharp increase in the susceptibility to cholesterol oxidase of human red blood cell and fibroblast plasma membranes that have been augmented slightly with cholesterol (7, 8). We interpret this effect to signify that, above the threshold, cholesterol membrane molecules are free of constraint by phospholipids and more frequently bob out of the plane of the bilayer, where the peripherally bound enzyme can access them (see Ref. 9). Similarly, the rate of transfer to cyclodextrin of membrane cholesterol in slight excess of normal is dramatically increased (2, 8). This effect could reflect a high frequency of transient projections of membrane sterol molecules into the aqueous phase where they can be captured by the acceptor (10). Finally, augmenting the plasma membranes of fibroblasts with a slight excess of cholesterol drives some to the endoplasmic reticulum, perhaps because of its high escape potential. Multiple regulatory elements in the endoplasmic reticulum sense the elevated cholesterol and respond by reducing the cell cholesterol level (8, 20).

These data suggest that a simple physicochemical mechanism plays a central role in the homeostasis of eukaryotic cell cholesterol: the steady-state level of the sterol in the plasma membrane is set by the capacity of its phospholipids (perhaps along with other relevant polar lipids). Passive and active adjustments made in response to the chemical potential of the cholesterol in excess of the equilibrium point serve to restore the resting level of plasma membrane cholesterol (2, 8).

There is evidence that the association of cholesterol with phospholipids can be perturbed so as to increase its chemical activity. In particular, it has been suggested that ceramides and diglycerides, physiologically membrane-intercalating amphipathic alcohols, displace cholesterol from bilayer phospholipids (11, 12). In addition, octanol not only stimulates the oxidation of plasma membrane cholesterol by cholesterol oxidase and its transfer to cyclodextrin but also sends the sterol to the endoplasmic reticulum (8, 13). We therefore sought to test the emergent hypothesis that these intercalating alcohols increase the chemical activity of cholesterol by displacing it from phospholipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Dioctanoyl-sn-glycerol and N-hexanoyl-D-erythro-sphingosine were obtained from Avanti. L-α-lyso(palmityl)phosphatidyl-α-erythro-sphingosine, egg yolk lysophosphatidylcholine, 25HC, cyclodextrin, octanol, and cholesterol oxidase (Streptomyces sp) were all from Sigma. We purchased 1α,2α-[3H]cholesterol from Amersham Biosciences. Blood was donated by a healthy human volunteer. Red blood cells were separated from the plasma and buffy coat and washed thrice with 10 mM NaCl, 5 mM NaPi (pH 7.5); HMG, hydroxymethylglutaryl; HPLC, high pressure liquid chromatography.

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2 The abbreviations and trivial names used are: cyclodextrin, methyl-β-cyclodextrin; ceramide, N-hexanoyl-D-erythro-sphingosine; diglyceride, 1,2-dioctanoyl-sn-glycerol; 25HC, 25-hydroxycholesterol; PBS, 150 mM NaCl, 5 mM NaPi (pH 7.5); HMG, hydroxymethylglutaryl; HPLC, high pressure liquid chromatography.
Results

Intercalator Activation of Red Cell Cholesterol Tested by Transfer to Cyclodextrin—We have shown that the rate of efflux of red cell membrane cholesterol to excess cyclodextrin is stimulated both by increasing the cholesterol to just above the physiologic level and by treatment with octanol (8). As shown in Fig. 1, ceramide and diglyceride also stimulate this transfer reaction. The apparent first-order rate constant for transfer was increased ~5-fold in both of the experiments shown.

The hypothesis under test predicts that it is not the rate of efflux of red cell cholesterol to cyclodextrin that should be increased by its displacement from phospholipids but rather the partition coefficient. We therefore determined the effect of the intercalators on the equilibrium distribution of cholesterol between red cells and cyclodextrin. Both ceramide and diglyceride were found to increase the partition to cyclodextrin by about 40% under specified conditions (not shown). The effect was predictably not large. This was because only the small fraction of cholesterol molecules that were active and not the bulk fraction would have a high escape potential. Furthermore, the amount of cyclodextrin used was minimized to avoid cell lysis. Finally, cholesterol partition might have been reduced adventitiously if the intercalators bound to the cyclodextrin as well as the membranes. Indeed, octanol binding to cyclodextrin could explain why it did not alter the equilibrium partition of cholesterol (data not shown), although octanol did speed cholesterol transfer in kinetic experiments (8).

Intercalator Activation of Red Cell Cholesterol Tested with Cholesterol Oxidase—The cholesterol in red cells is normally resistant to cholesterol oxidase; however, its susceptibility is greatly enhanced when bilayer cholesterol is increased slightly and also when the cells are treated with octanol (7, 13). Fig. 2 shows that treating red cells with three other membrane-intercalating alcohols, ceramide, diglyceride, and 25HC, also strongly increases the oxidation of their cholesterol. 25HC acted at less than a molar equivalence to the red cell cholesterol; ceramide and diglyceride were robust at molar equivalence. The action of the more water-soluble octanol required a ~20-fold excess over cholesterol (of which, presumably, only a small fraction was actually taken up). In all four cases, the stimulation curve was sigmoidal, reminiscent of the strong threshold effect characteristic of cholesterol concentration dependence (7, 8).

The oxidation of their membrane cholesterol caused red cells to lyse over time. This hemolytic action offered a convenient way to assess cholesterol oxidation. Fig. 3 shows that hemolysis reports with high sensitivity the same stimulation of cholesterol oxidation by the intercalators as that determined by chemical analysis in Fig. 2.

It has been proposed that the action of cholesterol oxidase depends on the chemical activity of cholesterol in the membrane, which, in turn, reflects the fraction of the sterol not associated with phospholipids (8). It follows that expanding the membrane phospholipid compartment should reduce the pool of active cholesterol and thereby inhibit cholesterol oxidase attack. This was observed with lysophosphatidylcholine, a convenient water-soluble membrane-intercalating phospholipid (13). If the mechanism by which intercalators stimulated the cholesterol oxidation action in Figs. 2 and 3 involves the displacement of cholesterol from phospholipids, augmenting the phospholipid pool with a slight excess of...
lysocephatidylcholine should inhibit the activity of cholesterol oxidase promoted by these agents. Fig. 4 supports this hypothesis.

**Intercalator Activation of Red Cell Cholesterol Tested with Saponin**—Various lytic agents act on cells in a cholesterol-dependent fashion; for example, the saponins (16, 17). We therefore tested whether the lysis of red blood cells by saponin depends on the activity of excess cholesterol. This seemed not to be the case since cells depleted of ~20% of their sterol (and consequently far below the threshold at which active cholesterol appears) could nevertheless be fully lysed (Fig. 5A). Rather, the concentration of saponin required for lysis varied inversely with membrane cholesterol. This behavior suggested a high-order mass-action relationship between the saponin and the sterol. If so, saponin might have to compete with phospholipids for association with the sterol. In that case, the intercalators under study should potentiate lysis by displacing cholesterol from the phospholipids. This premise is supported by the results shown in Fig. 5, B and C, for octanol, ceramide, and diglyceride. (Adding 25HC at levels far below that of the membrane cholesterol also promoted saponin hemolysis; however, this potent effect might simply reflect the formation of lytic complexes between 25HC and saponin.)

A further prediction of our hypothesis, arising from the findings presented in Figs. 4 and 5, is that introducing lysocephatidylchinoles into the membrane should counter the ability of the intercalating alcohols to promote hemolysis by saponin. We found this to be the case with octanol, diglyceride, and ceramide (data not shown). It therefore appeared that although saponin does not report on active cholesterol per se, it still provides a useful reagent for testing cholesterol displacement from membrane phospholipid.

**Substitution of Intercalators for Cholesterol in Cholesterol-depleted Red Cells**—It is well known that sterols such as cholesterol stabilize phospholipid bilayers, presumably through association with the aliphatic chains of the lipids (18, 19). If the displacement of sterols from phospholipids by intercalators reflects competition for phospholipid binding sites, they might substitute for the cholesterol removed from red cells by cycloheximide extraction. Fig. 6 supports this premise by demonstrating that octanol, ceramide, and diglyceride all arrested the hemolysis induced by depleting the membranes of cholesterol.

**Test of the Effect of Intercalators on Endoplasmic Reticulum Cholesterol**—Octanol has been observed to increase the level of endoplasmic reticulum cholesterol (20). However, its inhibitory action in this assay is also likely to involve its ability to stimulate the specific, ubiquitin-mediated proteolysis of the enzyme (21). On the other hand, the other three agents are not known to bind to HMG-CoA reductase, and they did not inhibit its activity when added to cell lysates (not shown but see Ref. 22). Furthermore, we have observed effect of 25HC is complex; it could reflect the known ability of 25HC to increase the level of endoplasmic reticulum cholesterol (20). However, its inhibitory action in this assay is also likely to involve its ability to stimulate the specific, ubiquitin-mediated proteolysis of the enzyme (21). On the other hand, the other three agents are not known to bind to HMG-CoA reductase, and they did not inhibit its activity when added to cell lysates (not shown but see Ref. 22). Furthermore, we have observed
that both octanol (8) and diglyceride\textsuperscript{3} increase endoplasmic reticulum cholesterol. Ceramide could not be tested because it inhibits the acyltransferase activity upon which the assay depends (23). As a check of the specificity of their action, we demonstrated that the inhibitory effects of octanol and diglyceride on HMG-CoA reductase activity were partially offset by reducing plasma membrane cholesterol in excess of the membrane lipids with which it can associate. Delineating a family of such compounds could aid in the study of the associations made between phospholipids, sterols, and membrane intercalators. Conversely, it would also be useful to identify agents that associate with sterols, as does lysophosphatidylcholine (Fig. 4), by screening for their ability to oppose the effects of cholesterol enrichment or of the intercalating alcohols examined above.

FIGURE 6. Intercalating agents inhibit the lysis of cholesterol-depleted cells. Red cell cholesterol was reduced to 45% of normal by incubating 20 μl of cells for 3 min at room temperature in 4 ml of PBS containing 1.5% cyclodextrin (see Ref. 8). The depleted cells were washed, and 1-μl aliquots were preincubated with 1% ethanol (C), 16 nmol of diglyceride (4), 8 nmol of ceramide (3), or 250 nmol of octanol (2) in 200 μl of PBS (final volume) in a 96-well plate. The optical absorbance at 500 nm was followed over time. (Note that the starting absorbance had decreased from an input value of ~1.2, reflecting lysis from cholesterol depletion before the time course began.)

FIGURE 7. Intercalators cause an acute reduction in hydroxymethylglutaryl-CoA reductase activity. Flasks of human fibroblasts preincubated overnight with medium containing lipoprotein-deficient serum to increase HMG-CoA reductase activity were incubated with agents or solvent (<1% ethanol) in 2 ml of medium for 1 h at 37 °C prior to disruption and assay. Enzyme activity per mg of protein is plotted relative to the untreated control. Octanol (oct), 800 μM; diglyceride (DG), 80 μM; ceramide (cer), 80 μM; 25HC, 12 μM. Error bars are S.D. for four or five experiments. The p values for octanol, diglyceride, and ceramide were 0.004, 0.002, and 0.006.

FIGURE 8. Hemolysis screens for effectors of red cell membrane cholesterol activity. Aliquots of red cells (0.9 μl bearing ~1.9 nmol of cholesterol) were incubated with agents in a final volume of 200 μl of PBS in a 96-well plate as in the previous figure. A, intercalators potentiate cholesterol oxidase. Cells were preincubated with intercalators (wells 1–9) and then with cholesterol oxidase (0.4 units, wells 1–8) for 2 h at 37 °C. Row a, diglyceride at 0, 2, 4, 8, 12, 20, 24, and 2.4 nmol/well. Row b, ceramide at 0, 0.5, 1, 1.3, 1.8, 2.2, 2.8, and 2.8 nmol/well. Row c, octanol at 4, 8, 12, 14, 16, 20, 24, and 24 nmol/well. B, intercalators potentiate saponin. Cells were preincubated with intercalators (wells 1–7). Saponin (4 μg/well) was then added to wells 1–7, and the plate was incubated for 0.5 h at room temperature. Row a, octanol at 0, 40, 80, 100, 120, 160, 200, and 200 nmol/well. Row b, diglyceride at 0, 0.5, 1, 2, 4, 8, 12, and 12 nmol/well. Row c, ceramide at 0, 8, 12, 16, 18, 20, 24, and 24 nmol/well. C, lysophosphatides inhibit cholesterol oxidation. Red cell cholesterol was increased by ~50% as described under “Experimental Procedures.” Enriched cells (rows a and b) or unmodified cells (row c) were preincubated with ethanol alone (wells 1 and 2) or lysophosphatides (wells 3–6) and then with cholesterol oxidase (0.4 units in wells 2–5) or PBS (wells 1 and 6) for 1.5 h at 37 °C. Row a, enriched cells plus lysophosphatidylcholine (0, 0.2, 0.3, 0.4, and 0.4 μg/well). Row b, enriched cells plus lysophosphatidylether (0, 0.2, 0.3, 0.4, and 0.4 μg/well). Row c, unmodified cells preincubated with 1% ethanol (wells 1 and 2), 0.4 μg of lysophosphatidylether (wells 3 and 4), and 0.4 μg of lysophosphatidylether (wells 5 and 6) before incubation with PBS (wells 1, 3, and 5) or cholesterol oxidase (wells 2, 4, and 6).

\textsuperscript{3}Y. Lange, J. Ye, and T. L. Steck, unpublished data.
would be free, would consequently have a high fugacity, and would therefore have a greater potential to exit the bilayer. The chemical activities and extents of the putative pools of phospholipid-associated and free membrane cholesterol species have yet to be measured directly. However, indirect tests showed an order of magnitude increase of sterol activity beyond a threshold that is taken to be the saturation point of the phospholipid. Two such phenomena are an increased rate of cholesterol efflux to cycloexextrin and an increased susceptibility of cholesterol to oxidation by cholesterol oxidase (2, 7, 8). Another indirect indicator of cholesterol activity is the dynamic rise of the cholesterol content of fibroblast endoplasmic reticulum in response to small increments in plasma membrane cholesterol (20). It may also be relevant that the θ-toxin of Clostridium perfringens binds with high affinity to phospholipid vesicles and to plasma membranes in which cholesterol is in slight excess of a threshold near the equimolar, physiological level (24, 25).

There are two competing mechanistic hypotheses for how membrane phospholipids modulate the chemical activity of cholesterol. One postulates that the bulky head groups of bilayer lipids such as phosphatidylcholine and sphingomyelin shield neighboring sterol molecules from unfavorable hydrophobic contacts with the aqueous phase (1, 3). In this model, sterol molecules need not interact directly with the phospholipids but are simply driven into juxtaposition by their aversion to water. Cholesterol molecules in excess of the umbrella of phospholipid heads would be exposed to the bulk water phase and therefore have a high chemical activity. In the original studies, the excess cholesterol formed microcrystals over a period of several days (1). In our short-term experiments, active cholesterol might have remained free in the bilayer, given the evidence that it interacted strongly with probes in the aqueous phase (cholesterol oxidase and cycloexextrin) as well as within the membrane (saponin).

The second hypothesis posits that membrane sterols and phospholipids associate as stoichiometric complexes (2, 4). Adding support to the original evidence for this model is the recent finding that the transfer of cholesterol to phospholipid bilayers is associated with a favorable enthalpy change, presumably reflecting van der Waals contacts and hydrogen bond interactions rather than a hydrophobic driving force (26). Furthermore, the associations of phospholipids with various steroids exhibit considerable specificity; see, for example, Refs. 27–29. On the other hand, sphingomyelin, a plasma membrane lipid with particular affinity for cholesterol, does not show stereospecificity in its interaction with cholesterol (30, 31). It may therefore be that the associations of the sterols and phospholipids, although strong and enthalpically driven, are not discrete, stereospecific or stoichiometric. Rather, the complexes may be irregular and short-lived, such as those predicted by a computer simulation (32). (These associations would nevertheless involve close contacts and the formation of liquid ordered clusters that would predispose to but would not necessarily result in phase separation of raft domains (33)). The relevant feature here, as in the first model, is that a given mixture of phospholipids would have a defined capacity for holding cholesterol, beyond which the chemical activity of the free sterol would increase sharply (2, 8). A recent molecular dynamics simulation supports this concept (34).

It follows that agents that free bilayer cholesterol of its association with phospholipids should increase its chemical activity. Such an action was recently inferred from demonstrations that ceramide and diacylglycerol potentiate the action of cholesterol-specific bacterial cytolysins (11) and displace cholesterol from rafts in synthetic bilayer vesicles (12). Also consistent with the cholesterol-displacement mechanism were our observations that octanol promoted the susceptibility of cholesterol to cholesterol oxidase and the efflux of cholesterol to cycloexextrin; furthermore, both octanol and 25HC evoked an increase in the cholesterol content of the endoplasmic reticulum of fibroblasts (8, 13, 20). To consolidate the emergent hypothesis, the present study tested four membrane-intercalating alcohols (octanol, ceramide, diacylglycerol, and 25HC) in a single, favorable system, the human red cell membrane. The assays were the efflux of cholesterol to cycloexextrin (Fig. 1), sensitivity to hemolysis by saponin (Fig. 5), and the susceptibility of cholesterol to cholesterol oxidase (Figs. 2–4 and 8A). In each test, the intercalator mimicked the effect of a slight excess of cholesterol (itself a membrane-intercalating alcohol).

That sterols can be displaced from their association with membrane phospholipids is a new concept. We offer as support the findings that non-steroid amphipaths substitute for extracted cholesterol in protecting the cells from lysis (Fig. 6) and that lysosphatidylcholines counter the ability of the intercalators to promote the action of cholesterol oxidase (Fig. 4 and 8C), just as they counter excess cholesterol (13). Presumably, the lysosphatidylcholines associate with the cholesterol displaced by the intercalators and thereby reduce its chemical activity, hence its susceptibility to the enzyme (8). (It is also conceivable that the lysosphatidylcholines associate to some degree with the intercalators to reduce their effect on cholesterol activity.) There is also calorimetric evidence for competition between octanol and cholesterol for association with phospholipid bilayers (35).

As a sterol derivative, 25HC presumably substituted for bilayer cholesterol isosterically, and because it might mimic as well as displace cholesterol, it was not of central importance in this study. Of more interest was the evidence that compounds as different from cholesterol as are diglycerides, ceramides, and octanol not only displace the sterol from phospholipids but substitute for it in maintaining bilayer integrity (Fig. 6). In keeping with the preceding discussion, we surmised that these intercalators, like cholesterol itself, do not make stereospecific complexes but rather that they enter into irregular associations with the phospholipids through hydrogen bonding of the head groups and van der Waals interactions among the hydrocarbon portions. These data encouraged the speculation that one of the several benefits conferred by the evolution of sterols could be their ability to oppose (buffer) variations in the properties of bilayers caused by the transient association of endogenous and exogenous intercalators with phospholipids. Looked at another way, a variety of endogenous and exogenous intercalators might be found to activate and/or substitute for sterols under various physiological and pathological circumstances. Ceramides are an example (12, 36).

Finally, our results have bearing on the mechanisms of cholesterol homeostasis. In the simplest case, the high escape potential of the molecules of plasma membrane cholesterol not associated with the phospholipids would, of itself, return the bilayer sterol to its resting level, a simple feedback mechanism. Indeed, in the case of the red blood cell, the physiological level of cholesterol hovers just below molar equivalence with the phospholipids, maintained by a passive collisional equilibrium with plasma lipoproteins (10, 37, 38). Similarly, the cholesterol in the plasma membranes of nucleated cells appears to seek a level that just saturates the phospholipids (8, 20). When the activity of the cholesterol in the plasma membrane exceeds the physiological threshold, the excess is disposed to move both to extracellular plasma lipoproteins and to intracellular membranes and possibly to binding proteins. Increased cholesterol in the endoplasmic reticulum would then signal the manifold regulatory elements therein to down-regulate the abundance of cell cholesterol through its esterification, the inhibition of its biosynthesis, and the down-regulation of the expression of genes for its accretion (8). Thus, although the upstream homeostatic signal arises from the physi-
cal chemistry of the plasma membrane bilayer, downstream responses are mediated by a multitude of intracellular proteins.

Similarly, in the case of membrane intercalators that displace cholesterol from its association with plasma membrane bilayer phospholipids, the high chemical activity of the displaced cholesterol would also increase the pool in the endoplasmic reticulum. This is how, for example, intercalators evoked the prompt inhibition of HMG-CoA reductase activity in Fig. 7. It is conceivable that, by this mechanism, certain membrane intercalators could be used to reduce hypercholesterolemia in afflicted individuals.

Acknowledgment—We thank Erwin London (State University of New York (SUNY), Stony Brook) for helpful discussions.

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