Loss of phosphoserine polar group asymmetry and inhibition of cholesterol transport in Jurkat cells treated with cholesterylphosphoserine

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Abstract Cholesterylphosphoserine (CPHS) is a synthetic ester of cholesterol showing immunosuppressive activity. In the present study, we have used the T cell line Jurkat to investigate its mechanism of action. CPHS incorporates into cells reaching a molar ratio of 0.23 and 3.9 with the total phospholipid and cholesterol content, without inducing necrosis or apoptosis. CPHS incorporation elicits a dose-dependent binding of fluorescein isothiocyanate-labeled annexin V, suggesting that the steroid distributes in the external leaflet of plasma membrane exposing the phosphoserine group to the external cell environment and inserting the steroid ring into the phospholipid bilayer. In agreement with a preferential steroid association with sphingolipids, CPHS is included in a Triton X-100-insoluble complex when mixed with sphingomyelin and cholesterol. CPHS incorporation inhibits the esterification of low density lipoprotein (LDL)-derived cholesterol, producing a minor influence on the endogenous synthesis of cholesterol and on the acyl-CoA:cholesterol acyltransferase activity. In this effect, CPHS is as potent as progesterone (IC_{50} of 3.5 μM). It is concluded that the insertion of cholesterylphosphoserine (CPHS) in the Jurkat plasma membrane neutralizes the asymmetric distribution of the phosphoserine group and inhibits the movement of cholesterol to the endoplasmic reticulum. As CPHS is a negatively charged steroid, this last effect may be linked to the perturbation of sphingolipid/cholesterol-based microdomains, proposed to play a role in cholesterol trafficking.—Cusinato, F., W. Habeler, F. Calderazzo, F. Nardi, and A. Bruni. **Loss of phosphoserine polar group asymmetry and inhibition of cholesterol transport in Jurkat cells treated with cholesterylphosphoserine. *J. Lipid Res.* 1998.** 39: **1844–1851.**

Supplementary key words progesterone • sphingolipid-based domains • caveolae • phospholipid asymmetry • annexin V • cholesterol transport

Specific lipid organizations contribute to the function of plasma membrane. The asymmetric distribution of phospholipids in the two leaflets of the bilayer provides the internal side with the negatively charged head groups, required to activate enzymes translocating from the cytosol. Also, lateral interactions between sphingolipids and cholesterol form close-packed clusters in the exoplasmic leaflet of plasma membrane, recruiting proteins involved in signal transduction (1). Depending on the association with a specialized protein called caveolin, the sphingolipid-based domains of plasma membrane are present in a cell as invaginations (caveolae) or flat glycosphingolipid-enriched rafts (2, 3). These two organizations have in common the property to be insoluble in the non-ionic detergent Triton X-100 and to lose their function upon cholesterol removal from the membrane. At variance with other cells such as fibroblasts, smooth muscle cells, endothelial and epithelial cells, lymphocytes, and T cell lines do not express caveolin (4). Therefore, caveolae cannot be detected in their plasma membrane. However, caveolin-free sphingolipid-based domains are operative in these cells mediating the endocytosis of glycosylphosphatidylinositol-anchored proteins, actin polymerization, and the recruitment of proteins implicated in T cell activation (1, 5).

In our attempts to affect T cell activation by altering plasma membrane lipid organization, we have previously tested phosphatidylserine, a phospholipid located on the inner leaflet of plasma membrane. The incorporation of phosphatidylserine into cells was expected to produce a reversible neutralization of phospholipid asymmetry, negatively influencing cell–cell communications and receptor function. Phosphatidylserine proved to be an effective immunosuppressant (6, 7) although its activity is dependent on a phosphatidylserine-specific phospholipase A1 found in fetal calf serum and human plasma, releasing the active metabolites 2-acyl-lysophosphatidylserine and unsaturated fatty acids (8). Following this line, we have synthesized a

Abbreviations: CPHS, cholesterylphosphoserine; BSA, bovine serum albumin; TBS, Tris-buffered saline; TPA, tetradecanoylphorbolacetate; ACAT, acyl-coA:cholesterol acyltransferase; LDL, low density lipoprotein; FITC, fluorescein isothiocyanate.

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novel serine phospholipid, cholesterylphosphoserine (CPHS), replacing diacyglycerol with cholesterol. Previous studies demonstrate that CPHS inhibits the proliferation of activated lymphocytes and, in mice, prevents the symptoms of delayed type of hypersensitivity and of graft-versus-host reaction (9, 10). These findings prompted the present investigation aimed at defining the mechanism of action of CPHS. The results show that CPHS incorporates in the T cell line Jurkat changing essential features of lipid organization in the plasma membrane such as the phospholipid head-group asymmetry and the composition of sphingolipid-based microdomains. A consequence of these effects is the inhibition of cholesterol transport from the plasma membrane to the endoplasmic reticulum.

MATERIALS AND METHODS

The sodium salt of cholesteryl-3-phospho-L-serine (mol wt 575.7) was dispersed at 1 mg/ml in 50% ethanol containing 5 mM Tris-HCl, pH 7.8, and briefly warmed at 50°C to obtain a clear solution which could be stored at 4°C for a week. The final CPHS concentration was monitored by phosphorus determination. 7-Ketocholesterol and progesterone (Sigma) were dissolved in ethanol. The influence of equivalent concentrations of ethanol in our tests was routinely checked.

Cell cultures

The human leukemic T cell line Jurkat was a gift from Dr. T. Pozzan (Department of Biomedical Sciences, University of Padova). The monoblastic-monocytic cell line U-937 was purchased from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia (Italy). Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Poiesys), 2 mM L-glutamine, and antibiotics. Jurkat cell cholesterol was determined in 5×10^7 cells after extraction with 20 volumes of chloroform–methanol 2:1 (v/v). The lipid extract, washed twice with 0.2 volumes of 0.1 M HCl, was taken to dryness and tested for total cholesterol content by a Sigma diagnostic kit based on the sequential action of cholesterol esterase and cholesterol oxidase. The same procedure of extraction followed by digestion with perchloric acid was used to determine the total phospholipid content. Two-dimensional thin-layer chromatography (11) indicated a phospholipid composition of 49% phosphatidylcholine, 25% phosphatidylethanolamine, and 6% phosphatidylserine, among the major phospholipids.

CPHS incorporation into cells

CPHS (0.1–0.2 μmol) was added to 10^7 Jurkat cells, suspended in 2 ml of a saline-buffered medium (TBS) composed of 120 mM NaCl, 5 mM KCl, 0.4 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mg/ml of bovine serum albumin (BSA), 25 mM Tris (pH 7.4). After 15 min of incubation at 37°C, the unbound CPHS was removed by cell sedimentation and washing. In the washing procedure the concentration of BSA in TBS was increased to 10 mg/ml to also eliminate the CPHS loosely adherent to the cells. The final cell sediment taken in 0.1 ml of 0.15 M NaCl was extracted with 20 volumes of chloroform–methanol 2:1 (v/v). The lipid extract was washed twice with 0.2 volumes of 0.1 M HCl. Separation of CPHS was obtained by two-dimensional thin-layer chromatography (plates of silica gel 60 from Merck). The chromatogram was developed twice in the first direction with chloroform–methanol–concentrated ammonia 65:25:4 (v/v) and once in the second direction with chloroform–methanol–acetic acid–water 50:30:8:4 (v/v). Two developments with the alkaline solvent were required to separate CPHS from the neutral phospholipids, phosphatidylcholine and sphingomyelin. The acidic run separated CPHS from phosphatidylserine which, like CPHS, had little mobility in the alkaline solvent (**Fig. 1**). CPHS was identified with a ninhydrin spray and quantified by phosphorus determination. Recovery was 100%.

Cell interaction with FITC-labeled annexin V

Two protocols were used. In the first, Jurkat cells (2×10^5 cells in TBS) were allowed to adhere for 1 h at 37°C on a glass coverslip covered with a film of 5 μg of immobilized human plasma fibronectin (Boehringer). To enhance the adhesion, 20 nM of tetradecanoylphorbolacetate (Sigma, TPA) was added during the cell incubation with fibronectin. The coverslips were washed and the adherent cells were incubated for 20 min at 37°C with 10 μM of CPHS. After washing, the cells were stained with fluorescein isothiocyanate (FITC)-labeled annexin V, using the Clontech ApoAlert Annexin V apoptosis kit. Fluorescent cells were observed in a Zeiss fluorescence microscope. Confocal microscopy of stained cells was performed in a Nikon-RCM 8000 real time confocal microscope. In the second protocol, Jurkat cells suspended in TBS (10^6 cells/ml) were treated with increasing CPHS concentrations for 30 min at 37°C, washed, and analyzed by flow cytometry (Epics Elite Coulter) after the addition of annexin V-FITC and propidium iodide to test fluorescence intensity and cytotoxicity at the same time. To evaluate apoptosis in these cells, the decrease of DNA content due to the endonuclease-induced formation of mono and oligo-nucleosomes was monitored by staining with propidium iodide after cell permeabilization with ethanol (12).

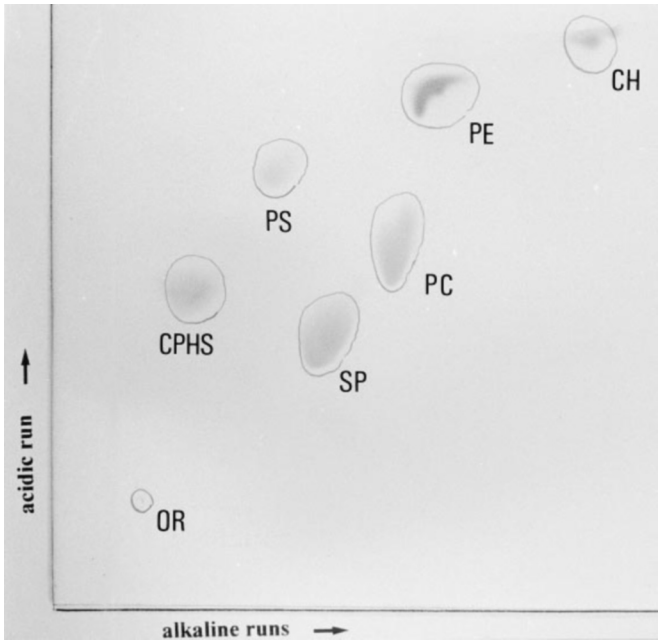

Fig. 1. Separation of CPHS from cholesterol and the major cell phospholipids in a two-dimensional thin-layer chromatography system. A mixture of known lipids (0.1 μmol each) was spotted at lower left. The chromatogram was first developed twice in the x-direction with chloroform–methanol–concentrated ammonia 65:25:4 (v/v) and then once in the y-direction with chloroform–methanol–acetic acid–water 50:30:8:4 (v/v). Spot identification: OR, origin; CPHS, cholesterylphosphoserine; PS, phosphatidylserine; SP, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Cholesterol (CH) migrated close to the front in both directions.

Cholesterol metabolism

The esterification of LDL-derived cholesterol was studied in Jurkat cells grown in RPMI containing 10% fetal calf serum. Cells were washed and resuspended (5×10^6 cells/ml) in RPMI containing 10% (v/v) of a low lipid serum replacement (Sigma, CPSR-1). Aliquots of 1 ml were supplemented with 5 μCi of [^{3}H]oleate (prepared in TBS free of divalent cations but containing 1 mg/ml of BSA) and incubated for 2 h at 37°C. Cells were then sedimented, washed, and resuspended in 0.1 ml of 0.15 M NaCl for lipid extraction. Cholesteryl esters were resolved in a monodimensional thin-layer chromatography system using petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v). Standards of cholesteryl oleate and triolein were included. Lipid spots were visualized with iodine, scraped, and quantified by scintillation counting. The incorporation of acetate in the lipid fraction was determined in the same way with the following variations. *a*) Cells were incubated for 18 h at 37°C in RPMI containing 5% low lipid serum replacement before the experiment. *b*) Ten μCi of [^{3}H]acetate was substituted for oleate. *c*) Plates were developed in hexane–diethyl ether–acetic acid 60:40:1 (v/v) together with the appropriate standards.

Detergent-insoluble complex of CPHS, cholesterol and sphingomyelin

Aliquots of 0.1 μmol each of bovine brain sphingomyelin (Sigma) and cholesterol from lanolin (5-cholesten-3β-ol, Fluka) with or without 0.1 μmol of CPHS, were mixed in chloroform–methanol 2:1 (v/v). The samples were taken to dryness under a stream of nitrogen and dispersed by vortex mixing in 0.1 ml of a saline medium composed of 120 mM NaCl, 5 mM KCl, and 25 mM Tris (pH 7.4). After a swelling period of 15 min at room temperature, the samples were supplemented with 0.9 ml of cold saline medium containing Triton X-100 (final concentration, 1%). After 30 min of incubation at 0°C, the samples were centrifuged in the cold 10 min at 10,000 rpm to remove the lipids that were not included in the insoluble complex. The sediment, collected in 0.1 ml of 0.5% sodium dodecylsulfate, was transferred to a glass tube, taken to dryness after repeated additions of chloroform–methanol 2:1, and analyzed by thin-layer chromatography using a double development in one direction with a solvent composed of chloroform–methanol–concentrated ammonia 65:25:4 (v/v). As phosphatidylserine was not present, a double development in the alkaline solvent was sufficient to clearly separate CPHS, sphingomyelin and cholesterol. Their resolution can be seen in Fig. 1. The spots corresponding to sphingomyelin and CPHS were quantified by phosphorus determination. Cholesterol was eluted and quantified by the Sigma diagnostic reagent kit.

RESULTS

CPHS incorporation into cells

Fifteen min incubation at 37°C of Jurkat cells in TBS with 10–20 nmol/10^6 cells of CPHS resulted in the incorporation of 2.47 ± 0.51 and 4.55 ± 0.55 nmol/10^6 cells, respectively (means ± SD from 6 independent experiments), corresponding to 23–24% of the added steroid. Extending the incubation to 30 min did not increase the CPHS incorporation. In agreement with the reported low values in leukemic lymphoid cells (13), we found a total cholesterol content in Jurkat cells of 1.17 ± 0.52 nmol/10^6 cells. The total phospholipid content was 19.6 ± 6.7 nmol/10^6 cells. Therefore, the amount of incorporated CPHS reached a molar ratio of 2.1 and 0.13 with respect to the cholesterol and the phospholipid content, respectively, when added at 10 nmol/10^6 cells and of 3.9 and 0.23 at 20 nmol/10^6 cells. When the cholesterol determinations were repeated in cells incubated with CPHS for 2 h, the measured cholesterol content did not change, indicating that CPHS was not hydrolyzed inside the cells or by the cholesterol esterase contained in the assay kit. In short times (1 h) of incubation in TBS, Jurkat cells remained viable after CPHS incorporation (trypan blue or propidium iodide exclusion tests). Afterward, cell death became gradually manifest. Provided the CPHS addition did not exceed 2 nmol/10^6 cells, cell death in prolonged incubations was prevented by the addition of 5% fetal calf serum or 10% of a low lipid serum replacement (Sigma, CPSR-1). When CPHS was preincubated for 15 min at 37°C with 20% of fetal calf serum before the contact with cells, the incorporation was reduced 70%. Extraction of CPHS after the incubation with fetal calf serum did not show hydrolysis of the compound, suggesting that the decreased incorporation was due to CPHS distribution in the serum lipoproteins.

Cell interaction with annexin V-FITC

To study whether the incorporated CPHS exposed its hydrophilic group toward the extracellular space, we took advantage of the property of annexin V to bind to phospholipids containing the phosphoserine group. As shown in **Fig. 2a**, TPA-treated Jurkat cells adhering to fibronectin stained positively with annexin V-FITC after a brief incubation with CPHS followed by washing. Analysis at higher magnification (Fig. 2b) showed that the CPHS–annexin complex distributed in all the cell surface, forming scattered patches of more intense fluorescence. Scanning confocal images (Fig. 2c) confirmed this observation and also showed that the CPHS–annexin V complex was not internalized during the time of observation (about 1 h). We used flow cytometry to study the binding of annexin V in cells not activated by the adhesion to fibronectin or by a TPA treatment. Cells in suspension were treated with CPHS and washed before the addition of annexin V-FITC. In the absence of a CPHS treatment, 63 ± 10% (n = 4) of the cells displayed a low fluorescence peak indicating a low annexin V-FITC binding. After treatment with CPHS, the appearance of a bright fluorescence peak comprising more than 99% of the cells indicated a dose-dependent increase of annexin V-FITC binding (**Fig. 3a**). The fluorescence emission in two separate experiments (number of activated channels) was 366–443 in the absence of CPHS treatment, and 490–566, 576–590, and 657–611 after treatment with 5, 10, and 20 μM of CPHS, respectively. When the impermeable dye propidium iodide was added at the end of the experiment, less than 1% of the cells were stained, indicating lack of cytotoxicity in all samples (data not shown). Because annexin V binding has been detected in apoptotic cells exposing phosphatidylserine (14), apoptosis in these samples was evaluated by determinations of DNA content. Cells were permeabilized with ethanol to allow the interaction of propidium iodide with

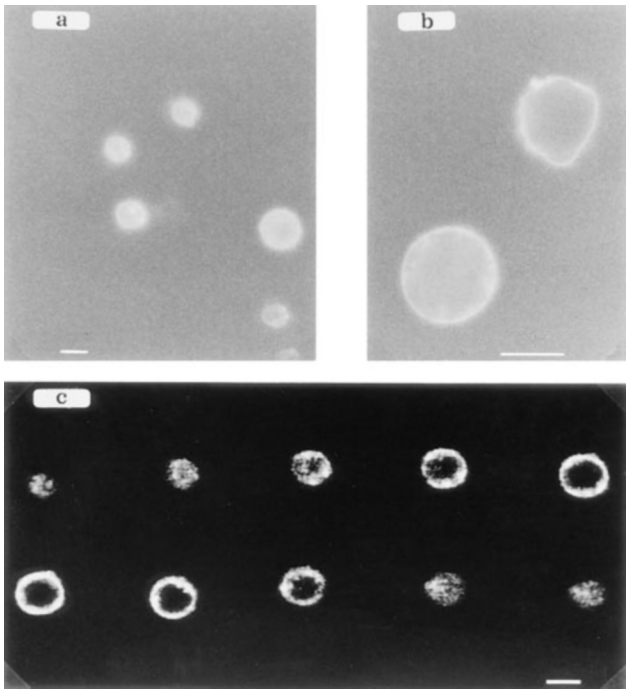

Fig. 2. CPHS-induced binding of annexin V to Jurkat cells. Cells in TBS supplemented with 20 nM of TPA were allowed to adhere on a film of 5 μg of fibronectin in a glass coverslip. After washing, cells were treated for 20 min at 37°C with 10 μM CPHS and washed again before the addition of annexin V-FITC. (a) and (b): Cell appearance in a fluorescent microscope at two different magnifications. (c): Fluorescent confocal images of a representative cell obtained by scanning from the upper to the lower surface. Calibration bars, 10 μm.

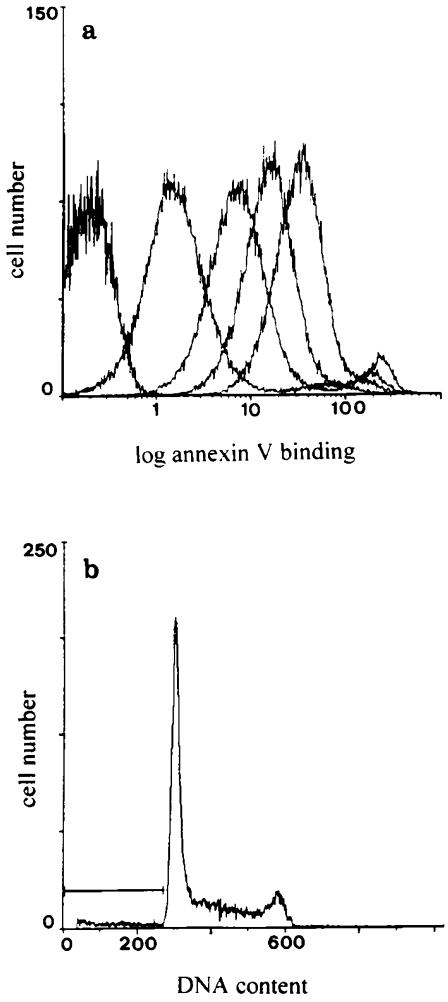

Fig. 3. Dose–effect relationship in the CPHS-induced annexin V binding. Jurkat cells in TBS (10^6/ml) were incubated for 30 min at 37°C with CPHS. After washing, the cell suspension was stained with annexin V-FITC and analyzed by cytofluorimetry. (a): Fluorescence peaks of stained cells. From left to right: cells not incubated with annexin V-FITC, cells incubated with annexin V-FITC without and after a treatment with 5, 10, and 20 μM CPHS. (b): DNA content of cells treated with 20 μM CPHS (the other samples showed the same pattern). The horizontal bar indicates the position of a fluorescence peak when the DNA content is decreased by the apoptosis-induced fragmentation. One of two similar experiments.

DNA (12). As shown in Fig. 3b for 20 μM CPHS, the cells at the end of the binding experiment with annexin V showed a single, homogeneous peak of fluorescence suggestive of unchanged DNA content.

Association of CPHS with sphingomyelin and cholesterol

If CPHS distributed in the exoplasmic leaflet of plasma membrane, its cholesterol moiety might show a preference for the association with the cell sphingolipids that are also concentrated in this region (15). To demonstrate this possibility we took advantage of the low solubility in Triton X-100 of the complex formed by sphingomyelin and cholesterol. Sphingomyelin and CPHS added separately or in combination to 1% Triton X-100 were solubilized by the detergent. In contrast, when cholesterol was also added, a precipitate was formed both in the presence and absence of CPHS. As CPHS is a charged amphiphile, its presence in the insoluble complex was manifest through a more dispersed appearance. In five experiments performed with 0.1 μmol of each lipid, the composition of the precipitates was (means ± SD) 71.5 ± 8.4 nmol of sphingomyelin and 59.7 ± 12.7 nmol of cholesterol in the absence of CPHS; 66.8 ± 6 nmol of sphingomyelin, 55.6 ± 14.7 nmol of cholesterol, and 47.4 ± 7.5 nmol of CPHS in its presence. The percent composition of precipitates was remarkably constant: 54.8 ± 3.4 of sphingomyelin and 45.1 ± 3.4 of cholesterol in the absence of CPHS; 39.6 ± 2.6 of sphingomyelin, 32.3 ± 3.8 of cholesterol and 28 ± 2.1 of CPHS in its presence.

Cholesterol metabolism

Next, we investigated the action of CPHS on the esterification of LDL-derived cholesterol. This process involves the transport of cholesterol from the plasma membrane to the sites of its esterification in the endoplasmic reticulum. Jurkat cells were grown in the presence of 10% fetal calf serum to load them with serum lipoproteins. Cells were then washed and incubated with labeled oleate in the presence of a low lipid serum replacement. CPHS did not affect oleate incorporation into triacylglycerols and

phospholipids (**Fig. 4a**) but significantly inhibited the labeling of cholesteryl esters (Fig. 4b). To determine whether the acyl-CoA:cholesterol acyltransferase (ACAT) activity was inhibited by CPHS, we tested the effect of this steroid in the presence of 7-ketocholesterol which is known to be an ACAT substrate (16). In three independent experiments, 7-ketocholesterol increased 5.6 times the incorporation of labeled oleate into cholesteryl esters in the absence of CPHS, 11 times with 5 μM CPHS, and 13.4 times with 10 μM. Therefore the inhibition by CPHS decreased from 62% to 26% at 5 μM and from 76% to 41% at 10 μM. To see whether the effect of CPHS was associated with the inhibition of the endogenous synthesis of lipids, Jurkat cells were depleted of serum lipoproteins by 18 h of incubation in a low lipid serum replacement. Labeled acetate was then added during 2 h of incubation. In five experiments, phospholipids collected 33% of total radioactivity and triacylglycerols collected 8%. Acetate incorporation in these lipids was not inhibited by 5 μM CPHS whereas at 10 μM we observed a partial but not significant decrease (**Fig. 5**). Cholesterol and cholesteryl esters collected 11 and 1.7% of total counts, respectively. Although a dose-dependent effect of CPHS was apparent on cholesterol synthesis, the values of inhibition were not significant.

The dose–response relationship in the CPHS-induced inhibition of cholesterol esterification is shown in **Fig. 6**. A comparison with progesterone, a reference steroid for the inhibition of cholesterol transport (17), is also presented. The IC_{50} for CPHS was 3.5 μM (0.7 nmol/10^6 cells in our conditions). Progesterone had the same potency. As a correlation has been proposed between steroid hydrophobicity and its efficacy in the inhibition of cholesterol esterification (18), we tested the hydrophobicity of CPHS and progesterone measuring their retention factor (R_f) using thin-layer chromatography with chloroform–methanol 12:1 (v/v) as a developing solvent (18). While progesterone had an R_f of 0.78, CPHS did not move from the origin.

To make a comparison with other cells, the experiments on cholesterol esterification were repeated with the monocytic cell line U-937. In three experiments, we observed that in these cells the incorporation of labeled oleate into the triacylglycerols was about twice that of Jurkat cells. Labeling of cholesteryl esters was in the same range. CPHS inhibited the incorporation of labeled oleate into cholesteryl esters by 53 ± 17% (mean ± SD) at 5 μM and 71.3 ± 5.8% at 10 μM. The incorporation of oleate into triacylglycerols was not affected.

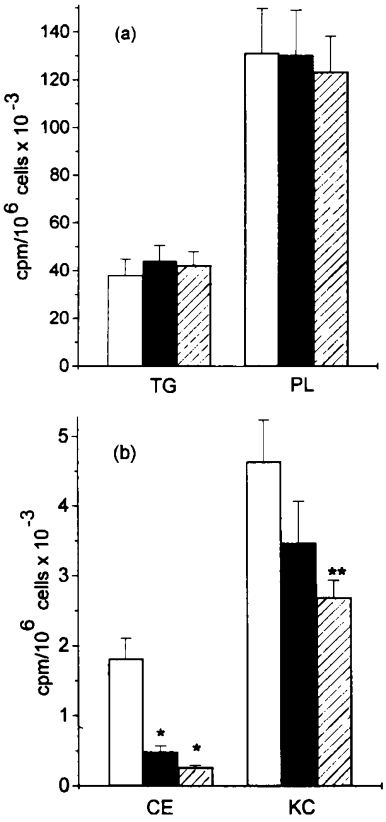

Fig. 4. Incorporation of [^{3}H]oleate into the lipid classes of Jurkat cells. Cells (5×10^6) were incubated for 2 h at 37°C in 1 ml of RPMI supplemented with 5 μCi of [^{3}H]oleate and 10% (v/v) of low lipid serum replacement in the absence (clear bars) or in the presence of 5 μM (solid bars) or 10 μM (cross-hatched bars) of CPHS. (a): Labeling of triacylglycerols (TG) and phospholipids (PL). (b): Labeling of cholesteryl esters (CE) and of cholesteryl esters when 10 μM of 7-ketocholesterol was also added to the incubation medium (KC). The data are means ± SEM from 10 independent experiments (3 experiments with 7-ketocholesterol).*$P < 0.001$, **$P < 0.05$.

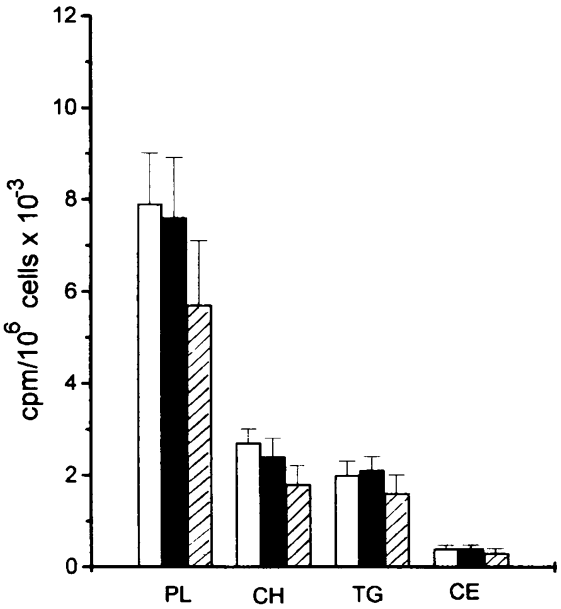

Fig. 5. Incorporation of [^{3}H]acetate into the lipid classes of Jurkat cells. Cells were depleted of serum lipoproteins by 18 h incubation in RPMI containing 5% (v/v) of a low lipid serum replacement. Cells (5×10^6) were then incubated for 2 h at 37°C in 1 ml of RPMI supplemented with 10% of a low lipid serum replacement and 10 μCi of [^{3}H]acetate in the absence (clear bars) or in the presence of 5 μM (solid bars) or 10 μM (cross-hatched bars) of CPHS. PL, phospholipids. CH, cholesterol. TG, triacylglycerols. CE, cholesteryl esters. The data are mean ± SEM from 5 independent experiments. The CPHS-induced changes were not significant.

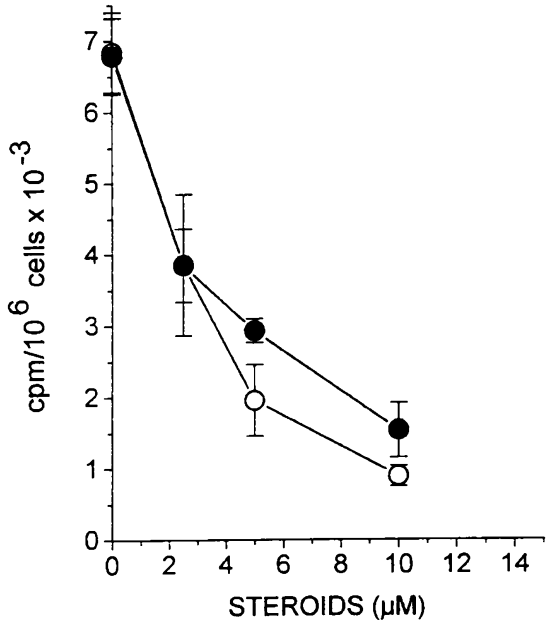

Fig. 6. Comparison between CPHS and progesterone in the inhibition of LDL-derived cholesterol esterification. The incorporation of [^{3}H]oleate into cholesteryl esters was determined as described in Fig. 4. CPHS (○), progesterone (●). Data are means ± SEM from 5 independent experiments. $P < 0.05$ at 2.5 μM; $P < 0.001$ at the other steroid concentrations.

DISCUSSION

This study shows that the synthetic cholesteryl ester CPHS incorporates into the plasma membrane of Jurkat cells neutralizing the physiological asymmetric distribution of the phosphoserine group and decreasing LDL-derived cholesterol esterification. While these results may be of relevance to the CPHS-induced inhibition of T cell activation (9, 10), they also show that this compound may become a reagent for the study of processes linked to the lipid organization of plasma membrane in a variety of other cells. Particularly, CPHS may help to clarify critical aspects of the bidirectional cholesterol transport between the plasma membrane and the intracellular organelles.

CPHS rapidly incorporates into the Jurkat T cell line. At the addition of 20 nmol/10^6 cells, 4.55 nmol/10^6 cells are incorporated without immediate manifestations of cytotoxicity. This amount is equivalent to 23% of total cell phospholipids and exceeds by almost 4 times the total cholesterol content. CPHS-treated Jurkat cells are enabled to bind annexin V-FITC, a protein showing affinity for the phosphoserine head group of phospholipids. The association with annexin V increases with the amount of incorporated CPHS and involves the totality of cell population. This fluorescent annexin V derivative has been used to show phosphatidylserine exposure in activated platelets (19) and in the early stage of apoptosis in B cells (14). In CPHS-treated cells, the exclusion of the impermeable dye propidium iodide and the absence of DNA fragmentation indicate that the binding of annexin V-FITC is not due to phosphatidylserine exposure as a consequence of CPHS-induced necrosis or apoptosis. In agreement, annexin V-FITC binding tests have been performed 30–60 min after a CPHS treatment whereas apoptosis in Jurkat cells becomes fully manifest 2–3 h after the addition of a specific apoptotic agent (20). From these data we conclude that annexin V interacts with the phosphoserine group of CPHS. This conclusion implies that part of the incorporated steroid distributes in the exoplasmic leaflet of plasma membrane, exposing the hydrophilic group outside the cell and inserting the steroid ring in the phospholipid bilayer. Adopting this position, CPHS neutralizes and even reverses the asymmetric distribution of the phosphoserine head group that, under physiological conditions, is only present in the internal leaflet of plasma membrane. It may be noted that CPHS incorporation may exceed the phosphatidylserine content of the cell (1.6 nmol/10^6 cells in our Jurkat cell line).

Bearing a bulky and hydrated polar group, CPHS is expected to slowly cross the plasma membrane. Therefore, its removal from the cell surface is dependent on plasma membrane recycling. In addition, a P-glycoprotein of the group encoded by the multidrug resistance genes may mediate its translocation to the inner surface of the membrane. This mechanism has been proposed for phospholipids (21) and cholesterol (18). Although CPHS internalization has not been investigated, we note that the images of confocal microscopy do not indicate a rapid internalization of the complex between CPHS and annexin V-FITC. This observation suggests that in the external leaflet of the plasma membrane CPHS may reach a concentration sufficient to perturb lateral lipid–lipid interactions. Due to the presence of a cholesterol moiety, CPHS might associate with sphingolipids. Thus, the microdomains formed by the aggregation of these lipids with cholesterol (1) might become a CPHS target. To test this possibility, we have examined the CPHS association with the Triton X-100-insoluble complex formed by sphingomyelin and cholesterol in a buffered saline solution. Early studies demonstrate that the sphingomyelin and the cholesterol of red cell membrane are resistant to extraction by this detergent (22). In addition, insolubility of these lipids in Triton X-100 is largely exploited to obtain the isolation of plasma membrane caveolae that are enriched in cholesterol and sphingolipids. In our tests we have first confirmed that Triton X-100 forms mixed micelles with sphingomyelin allowing a complete dispersion of this phospholipid (23). Being a charged amphiphile, CPHS is also solubilized by this detergent with or without sphingomyelin. In contrast, when cholesterol is added, Triton X-100 fails to solubilize sphingomyelin and CPHS, disclosing the propensity of CPHS to be associated with these lipids. As cholesterol is uncharged, it may provide the hydrophobic environment required to strengthen lipid–lipid interaction at the expense of lipid–detergent and lipid–water interaction. As suggested (24), this action involves the increase of van der Waals interactions between sphingomyelin molecules and the formation of hydrogen bonds between the cholesterol hydroxyl group and the amide group of the

sphingolipid. The association of CPHS with sphingomyelin and cholesterol is not marginal as we find that CPHS reaches 28% of the total lipids forming the precipitate when added at equimolar concentrations with respect to the other lipids.

Recent studies (reviewed in refs. 15, 25, 26) assign a role to the sphingolipid-rich regions of membranes in the transport of cholesterol from the trans-Golgi cisternae to plasma membrane and from the plasma membrane to the sites of its esterification in the endoplasmic reticulum. Although critical aspects of the intracellular cholesterol transport still await clarification, it is established that several lipid drugs, including the steroid U 18666A (27) and progesterone (17), act as inhibitors. According to the data obtained with the Jurkat cells, CPHS may be included in this list. In agreement with a CPHS-induced inhibition of the cholesterol transport from the plasma membrane to the endoplasmic reticulum, this steroid inhibits cholesterol esterification producing a marginal influence on the activity of acyl-CoA:cholesterol acyltransferase and on the endogenous synthesis of cholesterol. In this effect the potency of CPHS is equivalent to that of progesterone. The same observations have been made with the monocytic cell line U-937, indicating that the CPHS effect is not unique to Jurkat cells. The inclusion of the acidic CPHS among the steroids producing inhibition of cholesterol transport suggests that the molecule charge does not play a role in this effect as U 18666A is a basic compound and progesterone is neutral. Also, the greater CPHS hydrophilicity with respect to progesterone indicates that the presence of polar groups does not preclude this pharmacological effect. Rather, the polar moiety of CPHS may favor its accumulation in the plasma membrane and may increase the possibility of interaction with the sphingolipid/cholesterol microdomains that are membrane regions mediating the cholesterol efflux from the cells (28) and regulating its esterification (29). The high values of CPHS incorporation into the cells supports this possibility. More experiments are required to establish whether the inhibitory effect of CPHS results simply from the charge-induced disorder of these close-packed sphingolipid/cholesterol clusters or requires a direct interaction with an effector protein concentrating in these specialized membrane regions. Recent evidence points to the involvement of a P-glycoprotein (18) but other possibilities may be considered. For example, our experiments (F. Cusinato, M. Carrara, S. Bova, B. Visentin, and A. Bruni, unpublished results) demonstrate that CPHS prevents actin polymerization. The involvement of the actin cytoskeleton in the cholesterol esterification has been proposed in macrophages (30).

The CPHS-induced disorder in the lipid organization of plasma membrane may be the basis for the immunosuppressive activity produced by this steroid (9, 10). As the immune response relies on cell–cell communications, the presence of a negatively charged phosphoserine group in the external surface of lymphocytes may affect the activity of receptors mediating their contact with other cells. In addition, the CPHS interaction with the sphingolipid–cholesterol-based domains of plasma membrane may have negative consequences for the progress inside the cells of signals originated in the cell surface. The analysis of CPHS effect on T cells challenged with soluble and cellular stimuli will help to clarify this point.

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