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Cholesterylphosphoserine as inhibitor of cell adhesion and actin polymerization in human T cells

Federico Cusinato, Maria Carrara, Sergio Bova, Alessandro Bruni *

Department of Pharmacology, University of Padua, Largo Meneghetti 2, 35131 Padua, Italy

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Abstract

To further investigate the immunosuppressive activity of cholesterylphosphoserine (CPHS), we examined a variety of human T cell responses including proliferation, adhesion and cytoskeletal organization. The CPHS-induced inhibition of T cell response is greater in the integrin-dependent mixed lymphocyte reaction than in the integrin-independent proliferation elicited by anti-TCR-CD3 or anti-CD28 antibodies in the presence of tetradecanoylphorbol acetate. Consistently, CPHS inhibits the homotypic T cell adhesion involving the integrin αLβ2 (LFA-1) and the cell adhesion to fibronectin and rVCAM-1 involving the integrins of the β1 family. Since CPHS does not change integrin expression but inhibits post-receptor events such as cell spreading and pseudopodal projections, it seems likely that the site of CPHS influence is distal to the adhesion receptors. In agreement, the steroid prevents the reorganization of actin cytoskeleton occurring when T cells are allowed to spread on immobilized anti-CD3 in the absence of integrin activation. We suggest that CPHS acts on the metabolic pathway in which signals from integrin and growth factor receptors converge to induce the reorganization of the actin cytoskeleton. Selectivity in the action of CPHS is indicated by its ineffectiveness in the integrin-mediated adhesion of the monocytic cell line U-937 to fibronectin.

Keywords: Cholesterylphosphoserine; T cell; Cell adhesion; Integrin; Actin polymerization; Immunosuppression

1. Introduction

Currently used immunosuppressants act on a variety of cell functions including DNA synthesis, the assembly of transcription factors for the interleukin-2 gene and the enzymes regulating the cell cycle. Notable examples are mycophenolic acid, a selective inhibitor of type II isoform of inosine monophosphate dehydrogenase [1], the immunophilin-related drugs cyclosporin A and tacrolimus acting on the Ca^{2+}-dependent assembly of the nuclear factor of activated T cells [2], and sirolimus which inhibits the phosphorylation of p70 S6 kinase, required for the progression of T cells through the G_1 phase of the cell cycle [3]. Progress in understanding the T cell immune response suggests that plasma membrane may be an alternative target of immunosuppressants. Cell-cell recognition as well as the transmission of signals inside the T cells depend on the aggregation of surface receptors migrating within the plane of plasma membrane. In addition, key signaling pro-

Abbreviations: BSA, bovine serum albumin; CPHS, cholesterylphosphoserine; ICAM, intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule-1; PKC, protein kinase C; TBS, Tris-buffered saline solution; TdR, thymidine; TPA, tetradecanoylphorbol acetate

* Corresponding author. Fax: +39 (498) 275093; E-mail: bruni@ux1.unipd.it

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teins such as protein kinase C and Rho GTPases need to translocate to the plasma membrane in order to perform their function. On this basis it can be predicted that drugs changing the lipid organization of plasma membrane may affect these events. To this end steroid compounds seem suitable as their insertion into the plasma membrane is followed by an increase in lipid packing, influencing protein mobility and conformational changes [4]. In addition, they may interact with selected proteins. A preferential distribution of signaling proteins and steroids in the sphingolipid-cholesterol microdomains of plasma membrane [5] may favor this interaction. An example of steroid drugs acting in the plasma membrane is progesterone that inhibits the MDR glycoprotein-P [6] and the oxytocin receptor [7]. Following this line, we have developed a new synthetic cholesteryl ester, cholesterylphosphoserine (CPHS), that shows immunosuppressive activity [8,9]. More recently [10], we have shown that CPHS inserts its steroid ring into the T cell plasma membrane exposing the negatively charged phosphoserine group outside the cell. A consequence of CPHS insertion into the cell is the inhibition of cholesterol transport from the plasma membrane to the endoplasmic reticulum. These results suggest that CPHS may become a reagent to study T cell functions requiring the integrity of plasma membrane organization. A study has therefore been undertaken to investigate the effect of CPHS on the signaling activity of mitogenic and adhesive receptors. Our data show that a major consequence of CPHS insertion into the T cell membrane is the inhibition of the signaling pathway connecting these surface receptors to the organization of the actin cytoskeleton.

2. Materials and methods

2.1. Reagents

Monoclonal antibodies (mAbs) used were: anti-CD11a (anti-LFA-1), clone 38 and anti-CD3, clone UCHT1 from Ancell, anti-CD28, clone 15E8 from Cymbus Biotechnology, anti-CD49d (anti-VLA-4) clone SG/37 and anti-CD49e (anti-VLA-5) clone KH/33 from Seikagaku. The anti-CD 29 (anti-β1 integrins) was the BV7 mAb from Bioline [11]. Recombinant vascular cell adhesion molecule-1 (rVCAM-1) was a kind gift from Dr. R. Lobb (Biogen, Cambridge, MA, USA).

A saline buffered medium (TBS) used in some experiment had the following composition: 120 mM NaCl, 5 mM KCl, 0.4 mM $CaCl_2$, 2.5 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4). Cyclosporin A (Sandoz) was dissolved at 1 mg/ml in 50% ethanol. Cholesteryl-3β-phospho-L-serine (CPHS, Fig. 1), a generous gift from Dr. G. Kirschner (Fidia Research Laboratories, Padua, Italy) 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 verified by phosphorus determination. Cholesterol, cholesteryl acetate, cholesteryl-3β-phosphocholine (Fluka) and progesterone (Sigma) were dissolved and added in ethanol. The effect of the ethanol concentration added with all these compounds was routinely controlled.

2.2. Cell cultures

The human leukemic T cell line Jurkat, the human T lymphoma cell line SKW-3 and the human monoblastic-monocytic cell line U-937 were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and antibiotics. Peripheral blood mononuclear cells from healthy donors were isolated from heparinized venous blood by density gradient centrifugation using Ficoll-Paque. To deplete monocytes, T cells were separated by rosetting with neuraminidase-treated sheep erythrocytes, and further treated with 5 mM leucine methyl ester. The purity of the preparation was evaluated by the proliferation index obtained when one or two stimulating signals were applied to purified T cells. In our preparation the proliferation index increased approx. 10 times when 10 nM of tetradecanoylphorbol acetate (TPA) was added with 0.1 μg of immobilized anti-CD3 mAb. Non-specific cytotoxicity induced by CPHS in prolonged incubations was assessed by the trypan blue exclusion test and by the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) added to the cultures. To further evaluate a possible cell damage in the critical time of T cell activation (the first 24 h of incubation), 1 μCi of [^{3}H]oleate or [^{3}H]leucine was

added at the beginning of the cell culture. After 24 h, the energy-dependent incorporation of oleate into phospholipids or of leucine into cell proteins was determined.

2.3. T cell proliferation

Purified T cells (10^5) were incubated in microtiter plates at 37°C in 0.2 ml of RPMI medium containing 5% fetal calf serum (v/v), 1 mg/ml of bovine serum albumin (BSA), 2 mM L-glutamine, 50 μg/ml of transferrin, 100 U/ml of penicillin and 100 μg/ml of streptomycin. 1 μCi of [^{3}H]thymidine (TdR) was added in the last 18 h of culture. Cell activation was induced by 0.1 μg/well of immobilized anti-CD3 mAb (overnight incubation of the antibody added to wells at 4°C, followed by washing) or 0.2 μg/well of anti-CD28 mAb, each combined with 10 nM of TPA. Under these conditions the incubation time was 72 h at 37°C. Alternatively, T cell response was elicited by mixing 10^5 irradiated (3000 rad) allogeneic mononuclear cells with the same number of purified T cells in 0.2 ml of RPMI medium, supplemented as described above (mixed lymphocyte reaction). The incubation time was 6 days at 37°C.

2.4. Homotypic T cell adhesion

Purified T cells or SKW-3 cells (2×10^5 in 0.2 ml of TBS containing 2.5% fetal calf serum and 50 nM of TPA) were incubated with and without CPHS for 20 h at 37°C. Cell aggregation was then examined by an inverted microscope and photographed. The involvement of the integrin αLβ2 (LFA-1) in this process was tested by an antibody directed against the α subunit of the integrin (anti-CD11a). Quantitative evaluation of the CPHS effect was obtained by a computerized video imaging system coupled to an inverted microscope. Images of cell aggregates were acquired by a CCD camera, digitalized by an image processor and stored in a personal computer for analysis. To improve the quality of the digitalized pictures, each image was obtained by averaging 32 video frames. The area of cell aggregates was calculated in 25 fields by a custom-built software, and expressed as the number of pixels/field.

2.5. Adhesion and spreading on immobilized peptides

Wells of microtiter plates were coated with 2 μg of fibronectin or 0.2 μg rVCAM-1 according to established procedures, 1% BSA was used to block non-specific sites of adhesion. Jurkat or U-937 cells (10^6/ml) were preincubated for 30 min at 37°C in the absence and in the presence of CPHS in RPMI containing 10% (v/v) of a low-lipid serum replacement (Sigma CPSR-1) which had the advantage of avoiding CPHS dispersion into serum lipoproteins. Under this condition CPHS readily incorporated into the cells [10]. When indicated, TPA (20 nM) was also added during this preincubation to enhance the integrin-mediated cell adhesion [12]. After centrifugation and washing, 2×10^5 cells resuspended in RPMI containing 1 mg/ml of BSA were added to peptide-coated wells, and incubated for 60 min at 37°C. In the comparison between Jurkat and U-937 cells the concentration of BSA was increased to 10 mg/ml [13]. The wells were washed three times, and the attached cells were fixed with 2.5% glutardialdehyde in TBS. Adhesion was measured by staining the cells with 0.5% toluidine blue in 2.5% glutardialdehyde, and by reading at 630 nm, after dye extraction with 2% sodium dodecyl sulfate. 100% adherence, calculated by fixing the cells before washing, gave an absorbance value of 0.7–0.8. The spread morphology in fixed cells was evaluated using phase contrast microscopy. Alternatively, the adhesion was allowed to occur in a coverslip coated with fibronectin. The samples were postfixed with 1% osmic acid, dehydrated and critical point dried. After shadowing with Au-Pd, the samples were examined with scanning electron microscope.

2.6. Actin polymerization

The assay for the actin polymerization in adherent cells was performed as described [14]. Briefly, 2×10^5 control and CPHS-treated Jurkat cells in TBS containing 1 mg/ml of BSA were incubated for 10 min at 37°C on a glass coverslips coated (overnight incubation at 4°C followed by washing) with 1 μg of anti-CD3 mAb. The coverslips were washed, and the bound cells fixed for 30 min at 4°C with 2% paraformaldehyde in PBS. After 20 min permeabilization

with 0.1% Triton X-100 in PBS at 20°C, the coverslips were incubated for 45 min at 20°C in PBS containing 1% fetal calf serum. Cells were then exposed to 1 μg/ml of FITC-conjugated phalloidin (Sigma) for 20 min in the dark at room temperature. Coverslips were washed and observed in a Zeiss fluorescence microscope.

2.7. Protein kinase C activity

Jurkat cells (2.5×10^7) were incubated for 15 min at 37°C with 10 or 20 μM of CPHS in 10 ml of TBS supplemented with 1 mg/ml of BSA and 23 μM of leupeptin to avoid protein kinase C (PKC) degradation [15]. 20 nM of TPA was then added to initiate the translocation of PKC into the cell membranes, and the incubation followed for an additional 10 min. Cytosolic and membrane-associated PKC were extracted, partially purified on DEAE-cellulose, and assayed as described [16] with the following modifications: 40 μM leupeptin and 15 μM aprotinin were included in the lysis buffer; particulate PKC was extracted with 0.25% Triton X-100; the assay was conducted for 10 min at 37°C after the addition of 50 μM ATP containing 3 μCi of [γ-^{33}P]ATP (Amersham).

2.8. Lipid analysis

U-937 cells (10^7) were extracted with 20 vols. of chloroform-methanol 2:1 (v/v). The lipid extract, washed twice with 0.2 vol. 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. Phospholipids were resolved by TLC and quantified by phosphorus determination [10].

2.9. Flow cytometry

The expression of CD11a (the α subunit of LFA-1) in SKW-3 cells or CD29 (the β1 subunit of integrins) in Jurkat cells was measured by indirect immunofluorescence using a fluorescence-activated cell sorter (Epics Elite Coulter). Cells (10^6/ml) were pretreated with 10 μM of CPHS in the conditions described for the determination of homotypic cell adhesion (3 h of preincubation) or for the adhesion to fibronectin (30 min of preincubation). After centrifugation and washing with RPMI medium containing 1% (v/v) of fetal calf serum, cells were resuspended in the same medium at 7×10^6 cells/ml and incubated with the primary antibody (anti-CD11a or BV7) for 45 min at 0°C. After two washes, fluorescein-labeled goat anti-mouse IgG antibody was added and the cells incubated an additional 45 min. Controls were obtained omitting the primary antibody.

3. Results

3.1. T cell proliferation

To study the action of CPHS, we used two stimulating conditions for purified T cells. The first was a two-signal model of T cell activation, independent of the integrin-mediated cell-to-cell contact. The second was the integrin-dependent mixed lymphocyte reaction. To enhance CPHS incorporation, T cells were treated with the steroid and with the activating stimuli in the presence of 1 mg/ml of BSA. Fetal calf serum was added 10 min later. Since these experiments required a long period of incubation, several tests were performed to exclude non-specific, CPHS-induced cytotoxicity. Trypan blue exclusion and

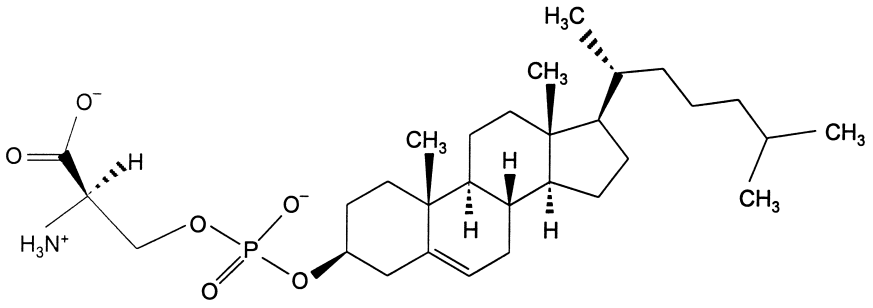

Fig. 1. Structure of cholesteryl-3β-phospho-L-serine.

MTT reduction tests performed at 24, 48 and 72 h of incubation showed that CPHS up to 10 μM did not cause loss of cell viability under the present conditions. Particularly, unchanged energy-dependent incorporation of [^{3}H]oleate into phospholipids or [^{3}H]leucine into proteins indicated that CPHS did not produce loss of high-energy compounds during the first 24 h of incubation, the time in which the signals from the surface receptors were transmitted to the T cell nucleus. We also considered the possibility that the CPHS-induced inhibitory effect on T cell proliferation was the consequence of inhibition of transferrin metabolism, previously documented with cholesterol [17]. To avoid this effect transferrin was included in the incubation medium.

As shown in Table 1, T cells proliferated when challenged with two signals activating a calcium-dependent pathway (immobilized anti-CD3 mAb in the presence of TPA) and a calcium-independent pathway (anti-CD28 in the presence of TPA). Under these conditions CPHS caused a significant but modest inhibition of T cell proliferation. As expected, an antibody (anti-CD11a), directed against the α subunit of the integrin αLβ2 (LFA-1) did not inhibit proliferation, confirming that the integrin-mediated cell-to-cell contact was not required. Although at a lower extent, purified T cells also proliferated when challenged with irradiated allogeneic mononuclear cells. This immune response was partially inhibited by the anti-CD11a mAb and completely by cyclosporin A. This result was consistent with the participation of LFA-1-mediated cell-to-cell adhesion in the mixed lymphocyte reaction [18]. In this model, the inhibition by CPHS was more evident and reproducible, reaching at 10 μM the same value as the inhibition produced by 5 μg/ml of the anti-CD11a mAb. This result suggested that the inhibition of cell adhesion mediated by the integrin LFA-1 played a part in the effect of CPHS. Comparative experiments with several steroids (not shown in Table 1) revealed that this effect was a distinctive property of CPHS. Cholesterol and cholesteryl acetate bearing a small head group in the 3β-position were inactive provided transferrin was added [17]. When the phosphoserine group was replaced by phosphocholine (cholesteryl-3β-phosphocholine), the cytotoxicity for the T cells increased, indicating that hydrophilicity and group size were critical. Among other steroids, progesterone that mimics the inhibitory action of CPHS on cholesterol transport to the intracellular sites of esterification [10], was likewise inactive.

3.2. Homotypic T cell adhesion

To further investigate the CPHS effect on the integrin function we examined the homotypic T cell

Table 1
Inhibitory effect of CPHS on lymphocyte proliferation

Addition	[^{3}H]TdR incorporation (cpm/culture $\times 10^{-3}$)	Inhibition (%)
1. None (12)	2.9 ± 0.5	
Immobilized anti-CD3, TPA (12)	78.2 ± 10.4	
10 μM CPHS (12)	52.5 ± 6.7*	31.6 ± 4.3
5 μg/ml anti-CD11a (3)	83.4 ± 2.5	
2. None (6)	2.8 ± 0.2	
Soluble anti-CD28, TPA (6)	77.4 ± 6.2	
10 μM CPHS (5)	50.6 ± 9.2*	37.8 ± 10.6
5 μg/ml anti-CD11a (3)	94.9 ± 3.7	
3. None (12)	3.4 ± 0.5	
Irradiated allogeneic cells (12)	20.4 ± 2.7	
10 μM CPHS (10)	9.7 ± 1.7**	50.6 ± 6.7
5 μg/ml anti-CD11a (5)	10.3 ± 1.4**	54.6 ± 6.3
0.8 μM cyclosporin A (4)	3.3 ± 0.6**	86.5 ± 1.5

Purified human T cell (10^5 cells in 0.2 ml of RPMI containing 1 mg/ml of BSA, 50 μg/ml of transferrin and antibiotics) were incubated for 10 min at 37°C in the presence of CPHS and the indicated additions (0.1 μg of immobilized anti-CD3; 0.2 μg of soluble anti-CD28; 10^5 irradiated allogeneic mononuclear cells; 10 nM of TPA). 5% (v/v) fetal calf serum was then added, and the incubation followed for 72 h (Expts. 1 and 2) or 6 days (Expt. 3). 1 μCi of [^{3}H]TdR was added in the last 18 h of culture. Means ± S.E.M. of the experiments indicated in parentheses, each performed in triplicate cultures. *$P < 0.05$; **$P < 0.01$.

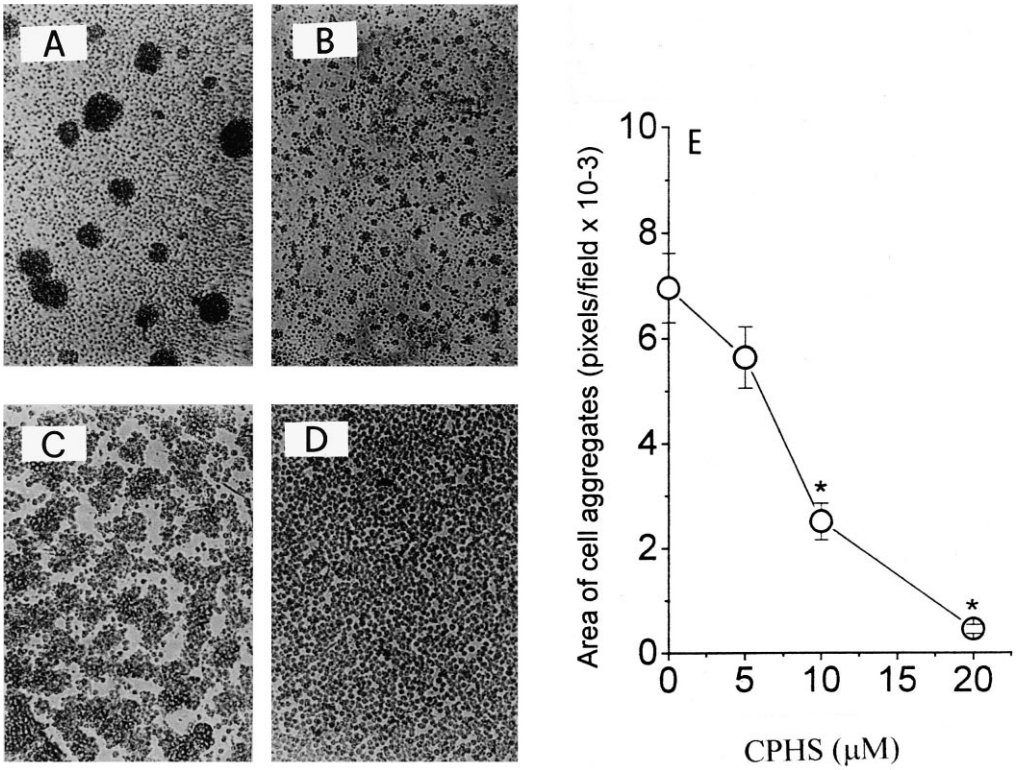

Fig. 2. Effect of CPHS on the homotypic T cell adhesion. Cells (2×10^5) in 0.2 ml of TBS containing 2.5% (v/v) fetal calf serum were incubated for 20 h at 37°C in the presence of 50 nM of TPA and, where indicated, 10 μM of CPHS. (A,B) Human T cells in the absence or in the presence of CPHS. (C,D) SKW-3 cells in the absence or in the presence of CPHS. In E the effect of increasing CPHS concentrations on the area of T cell aggregates was measured as described in Section 2. The data are expressed in pixels and represent the mean value of the number of pixels per field times 0.001, ±S.E.M. The asterisk denotes $P<0.05$. One of five experiments.

adhesion which reflects the interaction of the integrin LFA-1 with the intercellular adhesion molecules (ICAMs) in contiguous cells [19]. Purified human T cells incubated in the presence of fetal calf serum, Mg^{2+}, Ca^{2+} and TPA self-aggregated, gradually forming large clusters (Fig. 2A). This homotypic cell aggregation was prevented by the addition of 5 μg/ml of anti-CD11a mAb, indicating the involvement of the integrin LFA-1 and its interaction with ICAMs (data not shown). CPHS, like the anti-CD11a antibody, prevented the homotypic T cell adhesion (Fig. 2B). The same result was obtained with SKW-3 cells, a T cell line known [18] to express LFA-1 and the three ICAMs (Fig. 2C,D). To quantify the effect of CPHS, the area of the T cell aggregates was measured by a computerized video imaging system coupled to an inverted microscope (see Section 2). The results showed that the action of CPHS was dose-dependent with an IC_{50} of 8 μM (Fig. 2E). The inhibition of T cell aggregation was still present after 72 h of incubation. To study the CPHS influence on the integrin expression, we tested by flow cytometry the binding of anti CD11a mAb to SKW-3 cells which constitutively express a high level of LFA-1. In our conditions 79.8±1.2% of the SKW-3 cells bound the anti-CD11a mAb giving a mean fluorescence intensity of 361.5±4.6 ($n=3$). Pretreatment of the cells with 10 μM CPHS for 3 h either in the presence or absence of 50 nM TPA did not change these values.

3.3. T cell adhesion to fibronectin and rVCAM-1

To see whether CPHS was inhibitory to other integrin-mediated adhesions, we used the T cell line Jurkat, known to express the integrins of the β1 family involved in the adhesion of these cells to fibronectin and to the counter-receptor vascular cell adhesion molecule (VCAM-1). Since CPHS incorporated rapidly into these cells [10], its effect was examined on

cells shortly preincubated with the steroid (see Section 2). As shown in Fig. 3, CPHS inhibited the adhesion of Jurkat cells to fibronectin either with or without the TPA-induced enhancement of adherence [12]. In ten experiments the mean value of CPHS IC_{50} was 5.3 ± 2.9 μM. Although with lower potency, the inhibition by CPHS was also manifest in the adhesion of Jurkat cells to rVCAM. In this case, the CPHS IC_{50} was 8–10 μM in two experiments performed in the presence of TPA. Confirming previous data [12], we found that the cell adhesion to fibronectin is a rapid process. The maximal level of adherence by cells treated with TPA was reached in 20 min, and remained stable for at least 4 h. The inhibition by CPHS was fully manifest in the first 2 h, slowly declining thereafter, likely due to washout of the incorporated CPHS. Other steroids including cholesterol, cholesteryl acetate and progesterone were inactive (data not shown). Confirming the participation of β1 integrins in the adherence to fibronectin and rVCAM-1, a mAb (BV7) directed against the integrin β1 subunit inhibited the adhesion of Jurkat cells by 70–90%. A combination of antibodies directed against the α subunit of the integrins α4β1 and α5β1 inhibited by 60% the adhesion to fibronectin (data not shown). The BV7 mAb was selected to test by flow cytometry the influence of CPHS on β1 integrin expression. We found that 97.3 ± 0.6% of the cells bound the antibody with a mean fluorescence intensity of 440 ± 27 ($n = 3$). These values were not changed by a cell pretreatment with 10 μM CPHS, with or without the addition of 20 nM TPA.

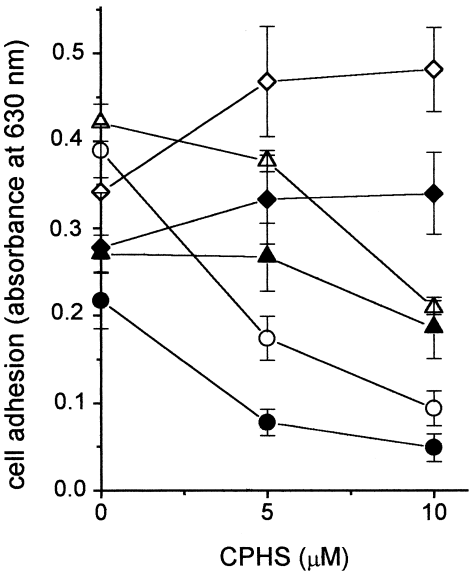

Fig. 3. Effect of CPHS on cell adhesion to immobilized fibronectin or rVCAM-1. Jurkat or U-937 cells were incubated for 30 min at 37°C with CPHS and, when indicated, 20 nM of TPA. After centrifugation and washing, 2×10^5 cells were added to wells coated with 2 μg of fibronectin or 0.2 μg of rVCAM-1 in 0.2 ml of RPMI supplemented with BSA as described in Section 2. Cell adherence was measured after 1 h at 37°C. Adhesion of Jurkat cells to fibronectin in the absence (●) or in the presence of TPA (○). Adhesion of Jurkat cells to rVCAM-1 in the absence (▲) or in the presence of TPA (△). Adhesion of U-937 cells to fibronectin in the absence (◆) or in the presence of TPA (◇). Data (means ± S.E.M.) are from ten experiments performed in triplicate (Jurkat, fibronectin), two experiments in duplicate (Jurkat, rVCAM-1) or four experiments in triplicate (U-937).

3.4. Adhesion of U-937 cells to fibronectin

To see whether CPHS influenced the integrin function in other cells, we tested the monoblastic-monocytic cell line U-937, known to adhere on fibronectin through the integrin α5β1 [13]. In agreement, we found that an antibody directed against the α subunit of the integrin α5β1 inhibited the adhesion of these cells to fibronectin by 90%, whereas an anti-α4β1 mAb was without effect (data not shown). Unexpectedly, CPHS failed to prevent the adhesion of U-937 cells to fibronectin, actually inducing stimulation (Fig. 3). To exclude that the CPHS ineffectiveness was due to insufficient steroid incorporation into the U-937 cells, a comparative analysis of CPHS incorporation was performed in Jurkat and U-937 cells [10]. In four experiments, the addition of 20 nmol of CPHS/10^6 cells caused the incorporation of 3.1 ± 2.2 nmol/10^6 Jurkat cells, a value close to that found in the previous experiments [10]. In the U-937 cells the incorporation was 4.8 ± 2.4 nmol/10^6 cells, corresponding to 24% of the added steroid. This observation indicated that the lack of CPHS-induced inhibition in U-937 cells cannot be ascribed to insufficient steroid incorporation. Since a substrain of U-937 cells has been reported to be deficient in cholesterol synthesis [20–22], we also tested the cholesterol content of our cells. Confirming the data on undepleted cells [20,22], we found that the total cholesterol content of our U-937 cells was 6.7 ± 0.6 nmol/10^6 cells and the total phospholipid content 25.2 ± 4.2 nmol/10^6 cells ($n = 4$). These values were the same after the

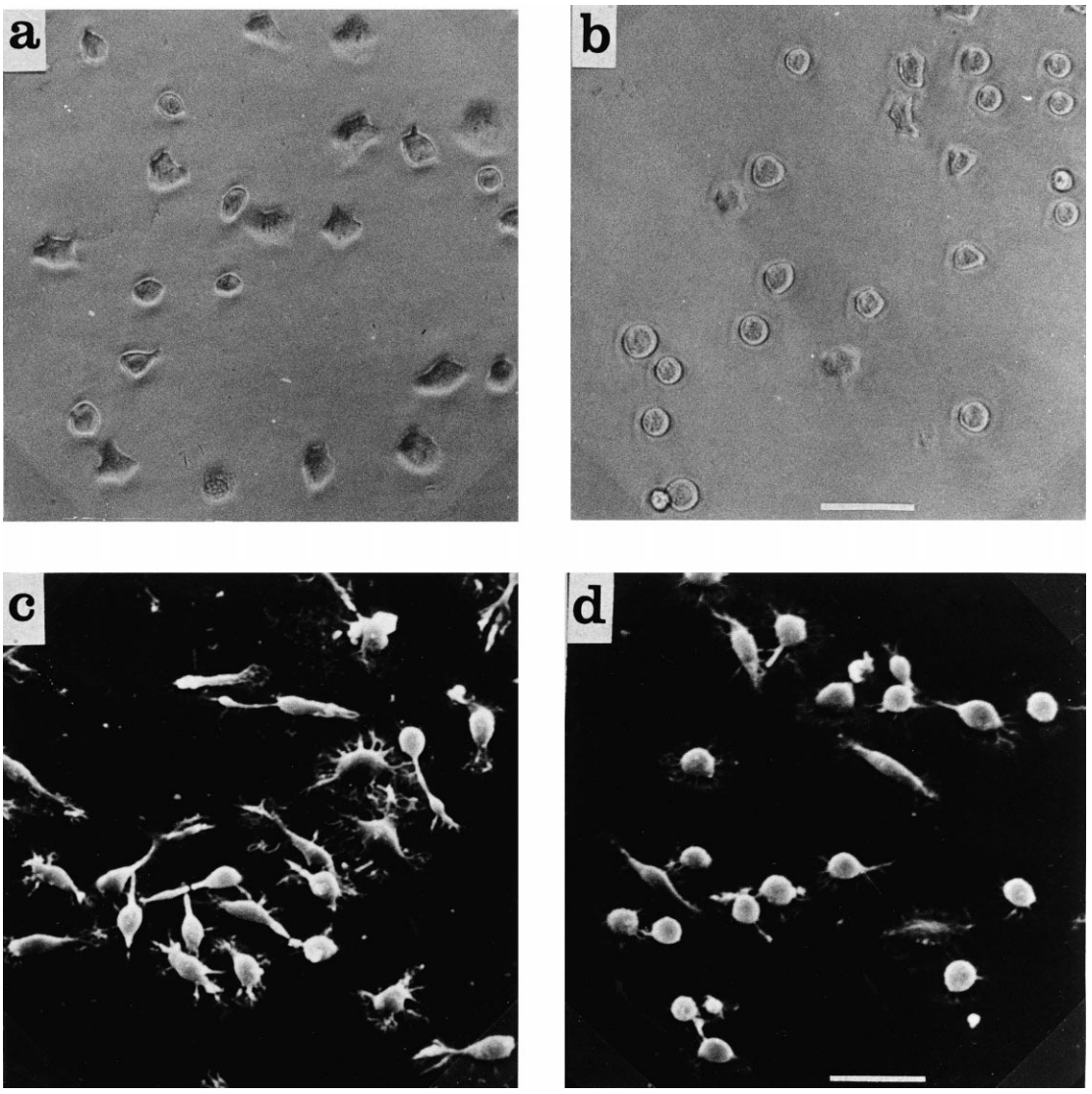

Fig. 4. Effect of CPHS on cell spreading. Control cells (2.5×10^4) or cells pretreated with 10 μM of CPHS were added to a film of immobilized fibronectin and incubated for 60 min at 37°C. Phase contrast images of adhering Jurkat cells without (a) and with (b) a pretreatment with CPHS. Scanning electron micrographs of adhering SKW-3 cells without (c) and with (d) a pretreatment with CPHS. Calibration bars, 50 μm (a,b), 20 μm (c,d).

short cell incubation in the absence of fetal calf serum needed to test the adhesion to fibronectin. In addition, we found that the value of total cholesterol content was the same in cells treated or not with CPHS, confirming [10] that this steroid was not hydrolyzed by the cholesterol esterases contained in the cells or in the kit used for the assay.

3.5. Organization of the actin cytoskeleton

As shown by the phorbol ester- and TCR-CD3-induced enhancement of cell adhesion without changes in the integrin expression or affinity [23–25], a main site of integrin regulation is located at post-receptor occupancy events. The activated signal sequence causes the reorganization of the actin cytoskeleton with cell spreading on the substrate and pseudopodal emission (reviewed in [26]). To see whether CPHS affected these processes, T cells adhering on fibronectin were fixed and examined by phase contrast microscopy or scanning electron microscopy. As shown in Fig. 4, the T cell lines, Jurkat

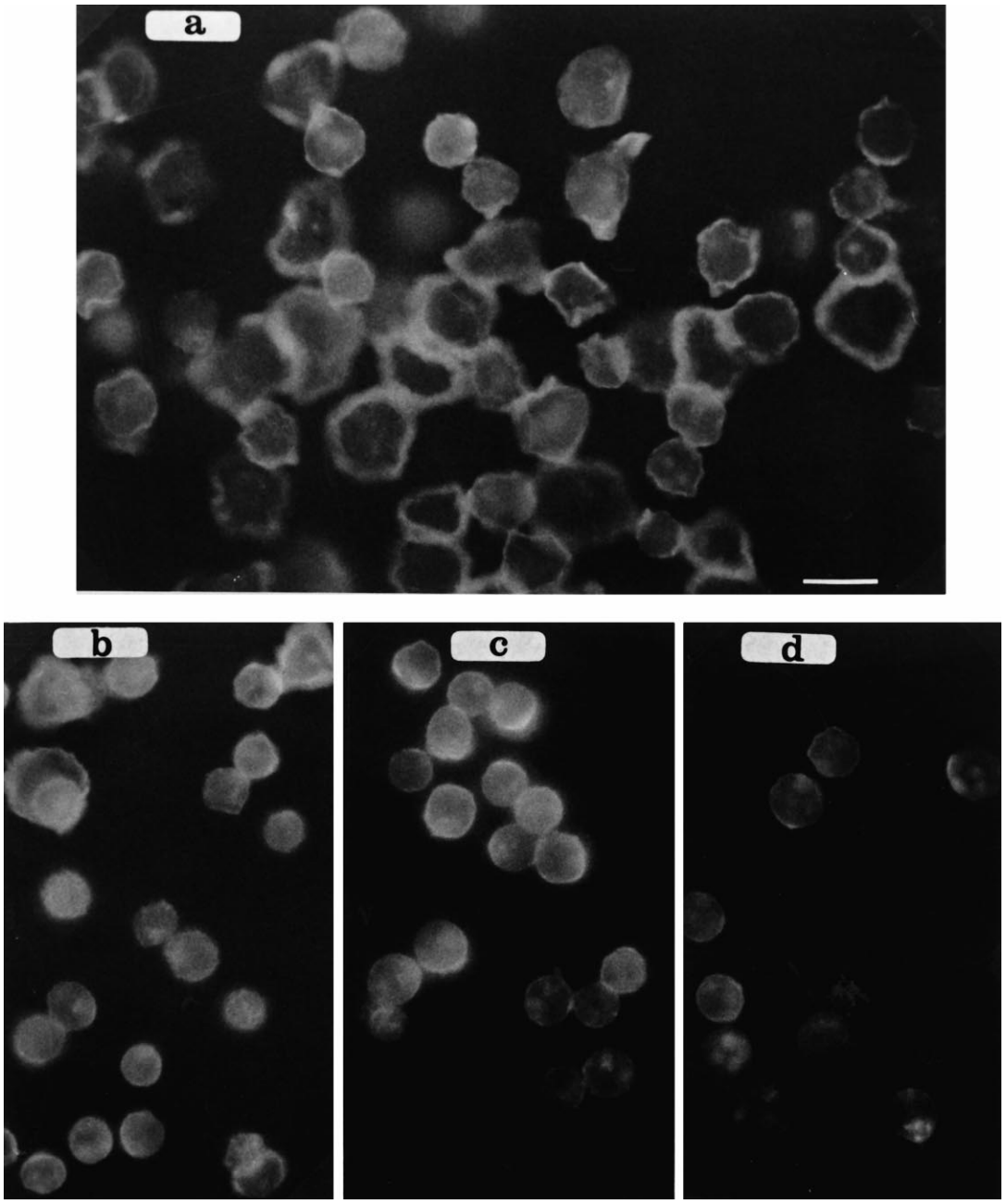

Fig. 5. Inhibition of actin polymerization in Jurkat cells adhering to anti-CD3 mAb. Cells were added to coverslips coated with 1 μg of anti-CD3 mAb and allowed to adhere for 10 min at 37°C. Cells were then washed, fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml of phalloidin-FITC. (a) Actin polymerization without a CPHS treatment. (b–d) Cells pretreated with 5, 10 and 20 μM of CPHS. Calibration bar, 20 μm. A representative of four similar experiments.

and SKW-3 adhering on fibronectin after CPHS treatment did not show a spread morphology and were less active in pseudopodal emission. This CPHS effect was still manifest examining the spread morphology after 2 h of adherence to fibronectin.

To see whether these CPHS effects were also manifest when the morphological changes did not originate from the integrin occupancy but occurred in response to intracellular events, we examined the adhesion of Jurkat cells to a film of immobilized anti-

CD3. To this end adherent cells were stained with phalloidin-FITC. Confirming previous observations [14], we found that Jurkat cells in the first 10 min of their adhesion to immobilized anti-CD3 mAb assembled a ring of polymerized actin close to the inner edge of the plasma membrane. At the same time the cells showed the spread morphology and irregular contours indicative of pseudopodal emission (Fig. 5a). The ring of polymerized actin was transient and not longer visible after 15 min [14]. After pretreatment with 5 μM of CPHS most of the cells retained the round morphology and failed to produce the ring of subcortical actin (Fig. 5b). Actin polymerization was further reduced at 10 μM of CPHS and almost abolished at 20 μM (Fig. 5c,d). To exclude that CPHS affected the cell binding to anti-CD3 we incubated Jurkat and purified T cells in microtiter plates coated with the immobilized antibody in a time interval between 10 min and 5 h. The pretreatment with CPHS did not inhibit the binding, although partial cell detachment on repeated washes (30%) indicated a weak adhesion.

3.6. Protein kinase C activity

One possible explanation of the CPHS effect on adhesion and actin polymerization was the inhibition of PKC activity. CPHS might interact with the PKC isoforms through its phosphoserine group. To study this possibility, CPHS-treated Jurkat cells were shortly incubated with TPA to induce PKC translocation. The PKC enzymes were then extracted and partially purified on DEAE-cellulose. In the absence of TPA addition, the PKC activity of the cells yielded 60 pmol of ^{33}P incorporated into the histone III-S/10^6 cells in 10 min of incubation. $93 \pm 8\%$ of this activity was distributed in the cytosol fraction ($n = 5$). After the addition of TPA, the membrane bound activity rose to 78%. Total activity and enzyme translocation were not changed by a 30 min pretreatment of the cells with 10 and 20 μM of CPHS.

4. Discussion

Extending previous observations [8] we have shown that the synthetic steroid CPHS negatively affects the proliferation of purified human T cells. To define the cellular mechanism of this effect, in this study we have employed two different stimulating conditions, namely T cell triggering by antibodies to CD3 and CD28 in the presence of a phorbol ester and the interaction with allogeneic cells (mixed lymphocyte reaction). The first model has been designed to test the effect of the steroid on the signaling pathways activated by two major mitogenic receptors under conditions excluding the integrin-mediated cell-to-cell contact. In addition, the stimulation through TCR-CD3 or CD28 in the presence of a phorbol ester provides a means to evaluate the role of intracellular Ca^{2+} in the effect of CPHS. Unlike the Ca^{2+}-sensitive T cell signaling pathway activated by TCR-CD3, the Ca^{2+}-independent pathway elicited by CD28 in the presence of a phorbol ester is not inhibited by cyclosporin A and tacrolimus [27]. The second model allowed to study the action of CPHS when the immune response requires the integrin-mediated T cell adhesion and spreading on a foreign cell. The data show that in the first model the inhibitory effect of CPHS, although significant, is modest. The similar effect after TCR-CD3 or CD28 stimulation indicates that the calcium sensitivity of the pathway is not influent. In contrast, the effect of CPHS becomes more manifest and reproducible in the mixed lymphocyte reaction. Since optimal T cell proliferation requires the provision of LDL cholesterol [28], we have first considered the possibility that the action of CPHS is the consequence of the steroid-induced inhibition of cholesterol transport shown before [10]. However, our comparative tests with progesterone, which mimics the action of CPHS on cholesterol transport but is ineffective on T cell proliferation, do not support this possibility. Considering the high steroid incorporation by the T cells [10], we therefore propose that CPHS acts by a perturbation of the plasma membrane biophysical properties resulting from the insertion of a steroid ring known to increase the rigidity of the lipid bilayer. In addition, the incorporated CPHS has been shown [10] to interact with the glycosphingolipid-cholesterol-rich membrane domains which are sites for the accumulation of many signaling molecules and for the initiation of actin polymerization [29,30]. These membrane changes may explain the reduced efficiency of signaling mechanisms, and are compatible

with the greater sensitivity to CPHS of the mixed lymphocyte reaction that involves the adhesive interaction between contiguous cells with the associated cell shape change and cytoskeletal reorganization [18,19,26]. In agreement, we find that a main CPHS-induced effect is the inhibition of the β1 and β2 integrin function. Considering the role of integrins in T cell interaction with the antigen presenting cells and in the lymphocyte extravasation [31], this CPHS effect may be relevant to the immunosuppressive activity shown in vivo by the steroid.

The evaluation of other steroids confirms that the plasma membrane may be the main target of CPHS effect. Progesterone, cholesterol and cholesteryl acetate, bearing an uncharged and small head group in the 3-position, may be rapidly internalized and transported to the intracellular sites of their metabolism. In accord, they are ineffective on T cell proliferation and adhesive properties. In contrast, CPHS, due to the presence of a hydrophilic head group, is not rapidly internalized [10], and may produce long-lasting effect in plasma membrane, as shown in this study. The role of a polar head has been further investigated by the comparison between CPHS and cholesteryl-3β-phosphocholine. This steroid has been selected because, like CPHS, it bears the head group of a membrane phospholipid (phosphatidylcholine). This allows the orientation of the steroid ring within the lipid bilayer, suited to reproduce the ordering effect of cholesterol [32]. The data show that the phosphoserine structure is preferable as a polar group since phosphocholine enhances the surfactant property of the amphiphile, causing cytotoxicity. Difference in size and hydrophilicity as well as the possibility of phosphoserine charge neutralization by extracellular cations may account for the distinct properties of two polar groups [33].

To investigate whether CPHS inhibits the integrin function by influencing the ligand binding site, we tested in Jurkat and SKW-3 cells the integrin expression using inhibitory antibodies. Antibodies abrogating the integrin function have been reported to prevent the binding of soluble ICAM-1 to LFA-1 [24] and of fibronectin to β1 integrins [25]. The data show that CPHS does not influence the binding of antibodies, suggesting that the integrin ligand binding site is not affected. This conclusion has been confirmed using the monocytic cell line U-937 where, at a comparable steroid incorporation, CPHS does not inhibit the integrin-mediated adhesion to fibronectin, actually causing a stimulation. We made determinations of cholesterol content in these cells to see whether the stimulating effect of CPHS results from a restoration of a cholesterol deficiency. U-937 cells are known to be sensitive to their cholesterol content. Membrane-linked functions, including endocytosis [20], the appearance of the adhesive phenotype after the phorbol ester-induced differentiation [21] and the PAF-stimulated Ca^{2+} mobilization [22] are severely reduced in a cholesterol auxotroph strain of these cells incubated for 24–48 h in the absence of serum lipoproteins. These functions are restored by a supply of exogenous cholesterol. At variance, our data show that the U-937 cells used in this study had a normal cholesterol content, not decreased by the short incubation in a lipoprotein-free medium needed to test the adhesion to fibronectin.

Two observations help to define the site of CPHS influence in T cell adhesion. First, CPHS inhibits cell spreading and pseudopodal projections which are events following the integrin occupancy. Second, the CPHS-induced inhibition of cell spreading and pseudopodal organization is also manifest when these processes are not initiated by the integrin occupancy, but originate from the inside-out signals triggered by the engagement of TCR-CD3. In this condition we find that CPHS inhibits the assembly of polymerized actin underlying cell spreading and pseudopodal emission. These results suggest that the site of CPHS inhibition is distal to the adhesion receptors, and can be located in the metabolic sequence in which signals from the growth and the adhesive receptors converge to initiate the reorganization of the actin cytoskeleton [26]. This conclusion is consistent with the membrane changes induced by CPHS, negatively influencing the connection with the actin cytoskeleton.

A final question is whether CPHS causes its effect solely by a perturbation of the lipid organization or whether a defined signaling protein is involved. As a first attempt to address this point, we have tested the action of the steroid on protein kinase C. The results demonstrate that neither the activity nor the translocation of this signaling protein is influenced by CPHS. Instead, an indication of selectivity has been obtained in the comparison between the T cell line

Jurkat and the monocytic cell line U-937 since in the former CPHS inhibits the integrin-mediated adhesion to fibronectin whereas in the latter it causes a slight stimulation. This action of CPHS is reminiscent of the effect of C3-transferase from *Clostridium botulinum*, a specific reagent for the small GTP-binding Rho protein. C3-transferase prevents in T cells the homotypic cell-to-cell adhesion [34] but in the U-937 cells it enhances the adhesion to fibronectin [13,35]. This difference has been attributed to a distinctive effect of the Rho GTPases, Rho, Rac and Cdc42, on the organization of the actin cytoskeleton depending on the cell type [35,36]. The different response of T cells and U-937 cells to CPHS may be a first indication that the function of the Rho GTPases is particularly sensitive to the lipid organization imparted by the steroid. This possibility is now under investigation in our laboratory.

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References

[1] S.F. Carr, E. Papp, J.C. Wu, Y. Natsumeda, Characterization of human type I and type II IMP dehydrogenases, J. Biol. Chem. 268 (1993) 27286–27290.
[2] G.R. Crabtree, N.A. Clipstone, Signal transmission between the plasma membrane and nucleus of T lymphocytes, Annu. Rev. Biochem. 63 (1994) 1045–1083.
[3] N. Terada, R.A. Franklin, J.J. Lucas, J. Blenis, E.W. Gelfand, Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of p70 S6 kinase, J. Biol. Chem. 268 (1993) 12062–12068.
[4] P.L. Yeagle, Modulation of membrane function by cholesterol, Biochimie 73 (1991) 1303–1310.
[5] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[6] J.M. Ford, W.N. Hait, Pharmacology of drugs that alter multidrug resistance in cancer, Pharmacol. Rev. 42 (1990) 155–199.
[7] E. Grazzini, G. Guillon, B. Mouillac, H.H. Zingg, Inhibition of oxytocin receptor function by direct binding of progesterone, Nature 392 (1998) 509–512.
[8] A. Bruni, L. Mietto, F.E. Secchi, C. Chizzolini, Lipid mediators of immune reactions: effect of a linked phosphorylserine group, Immunol. Lett. 42 (1994) 87–90.
[9] A. Bruni, L. Mietto, F.E. Secchi, D. Ponzin, G. Monastra, G. Kirschner, F. Cusinato, C. Chizzolini, Inhibition by cholesterylphosphorylserine of T cell-mediated immune responses in mice, Int. J. Immunopharmacol. 17 (1995) 517–521.
[10] F. Cusinato, W. Habeler, F. Calderazzo, F. Nardi, A. Bruni, Loss of phosphoserine polar group asymmetry and inhibition of cholesterol transport in Jurkat cells treated with cholesterylphosphoserine, J. Lipid Res. 39 (1998) 1844–1851.
[11] I. Martin-Padura, G. Bazzoni, A. Zanetti, S. Bernascono, M.J. Elices, A. Mantovani, E. Dejana, A novel mechanism of colon carcinoma cell adhesion to the endothelium triggered by β1 integrin chain, J. Biol. Chem. 269 (1994) 6124–6132.
[12] J.A. Wilkins, D. Stupack, S. Stewart, S. Caixia, β1 Integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment, Eur. J. Immunol. 21 (1991) 517–522.
[13] M. Aepfelbacher, ADP-ribosylation enhances adhesion of U-937 cells to fibronectin via the α5β1 integrin receptor, FEBS Lett. 363 (1995) 78–80.
[14] M.V. Parsey, G.K. Lewis, Actin polymerization and pseudopod reorganization accompany anti-CD3-induced growth arrest in Jurkat cells, J. Immunol. 151 (1993) 1881–1893.
[15] K. Chida, N. Kato, T. Kuroki, Down regulation of phorbol diester receptors by proteolytic degradation of protein kinase C in a cultured cell line of fetal rat skin keratinocytes, J. Biol. Chem. 261 (1986) 13013–13018.
[16] J.A. Ledbetter, C.H. June, P.J. Martin, C.E. Spooner, J.A. Hansen, K.E. Meier, Valency of CD3 binding and internalization of the CD3 cell-surface complex control T cell responses to second signals: distinction between effects on protein kinase C, cytoplasmic free calcium, and proliferation, J. Immunol. 136 (1986) 3945–3952.
[17] J.A. Cuthbert, P.E. Lipsky, Immunoregulation by low density lipoproteins in man: inhibition of mitogen-induced proliferation by interference with transferrin metabolism, J. Clin. Invest. 73 (1984) 992–1003.
[18] A.R. de Fougerolles, X. Quin, T.A. Springer, Characterization of the function of intercellular adhesion molecule ICAM-3 and comparison with ICAM-1 and ICAM-2 in immune response, J. Exp. Med. 179 (1994) 619–629.
[19] R. Rothlein, T.A. Springer, The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester, J. Exp. Med. 163 (1986) 1132–1149.
[20] M. Esfahani, L. Scerbo, S. Lund-Katz, D.M. DePace, R. Maniglia, J.K. Alexander, M.C. Phillips, Effects of cholesterol and lipoproteins on endocytosis by a monocytic-like cell line, Biochim. Biophys. Acta 889 (1986) 287–300.
[21] M. Esfahani, S. Hathotuwegama, A. Kalenak, L. Scerbo, H.M. Brown, A requirement for cholesterol for phorbol ester-induced adhesion of a human monocyte-like cell line, Biochem. Biophys. Res. Commun. 144 (1987) 1167–1174.

[22] M. Aepfelbacher, N. Hrboticky, I. Lux, P.C. Weber, Cholesterol modulates PAF-stimulated Ca^{2+}-mobilization in monocytic U937 cells, Biochim. Biophys. Acta 1074 (1991) 125–129.
[23] Y.N. Danilov, R.L. Juliano, Phorbol ester modulation of integrin-mediated cell adhesion: a postreceptor event, J. Cell Biol. 108 (1989) 1925–1933.
[24] M.P. Stewart, C. Cabanas, N. Hogg, T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1, J. Immunol. 156 (1996) 1810–1817.
[25] R.J. Faull, N.L. Kovach, J.M. Harlan, M.H. Ginsberg, Stimulation of integrin-mediated adhesion of T lymphocytes and monocytes: two mechanisms with divergent biological consequences, J. Exp. Med. 179 (1994) 1307–1316.
[26] B.M. Gumbiner, Cell adhesion: the molecular basis of tissue architecture and morphogenesis, Cell 84 (1996) 345–357.
[27] N.H. Sigal, F.J. Dumont, Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction, Annu. Rev. Immunol. 10 (1992) 519–560.
[28] J.A. Cuthbert, P.E. Lipsky, Modulation of human lymphocyte response by low density lipoproteins (LDL): enhancement but not immunosuppression is mediated by LDL receptors, Proc. Natl. Acad. Sci. USA 81 (1984) 4539–4543.
[29] T. Harder, K. Simons, Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains, Curr. Opin. Cell Biol. 9 (1997) 534–542.
[30] M. Deckert, M. Ticchioni, A. Bernard, Endocytosis of GPI-anchored proteins in human lymphocytes: role of glycolipid-based domains, actin cytoskeleton and protein kinases, J. Cell Biol. 133 (1996) 791–799.
[31] J.P. Girard, T.A. Springer, High endothelial venules (HEVs): specialized endothelium for lymphocyte migration, Immunol. Today 16 (1995) 449–457.
[32] M. Lyte, M. Shinitzky, Cholesteryl-phosphoryl-choline in lipid bilayers, Chem. Phys. Lipids 24 (1979) 45–55.
[33] A. Bruni, P. Palatini, Biological and pharmacological properties of phospholipids, Prog. Med. Chem. 19 (1982) 111–203.
[34] T. Tominaga, K. Sugie, M. Hirata, N. Morii, J. Fukata, A. Uchida, H. Imura, S. Narumiya, Inhibition of PMA-induced, LFA-1-dependent lymphocyte aggregation by ADP ribosylation of the small molecular weight GTP binding protein, Rho, J. Cell Biol. 120 (1993) 1529–1537.
[35] M. Aepfelbacher, M. Essler, E. Huber, A. Czech, P.C. Weber, Rho is a negative regulator of human monocyte spreading, J. Immunol. 157 (1996) 5070–5075.
[36] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.