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Jatrophone and 12-*O*-tetradecanoyl phorbol-13-acetate antagonism of stimulation of natural killer activity and lymphocyte proliferation

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Abstract

We have recently reported that the diterpene jatrophone antagonizes the effects of phorbol ester in pharmacogical studies. In order to investigate further whether this action is associated with an inhibition of protein kinase C activity, we examined the effect of jatrophone on the stimulation of lymphocyte activities which are dependent on the protein kinase C pathway. Jatrophone (0.02–0.32 μM) caused concentration-dependent and equipotent inhibition of human lymphocyte proliferation induced by 5 μg/ml of phytohemagglutinin or by a combination of 100 ng/ml of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) plus 0.15 μM ionomicyn, with IC_{50} values (and their 95% confidence limits) of 53.4 (42.6–65.3) nM and 48.4 (39.4–59.8) nM, respectively. Jatrophone also blocked, in a concentration-dependent fashion, the murine lymphocyte proliferation stimulated by 5 μg/ml of concanavalin A, with an IC_{50} value of 63.5 (51.2–76.5) nM. The inhibition was not due to a toxic effect as the pre-incubation of lymphocytes for 48 h with 0.32 μM jatrophone did not impair the proliferation after removal of the diterpene from the culture medium. Human lymphocytes when pre-treated with 10 ng/ml TPA had a 3 times higher spontaneous natural killer activity against K_{562} cells and an increased expression of CD69. In addition, jatrophone inhibited both spontaneous and TPA-stimulated natural killer activity and the expression of CD69. Jatrophone concentrations that inhibited 75% of lymphocyte proliferation did not impair the intracellular increase in Ca^{2+} flux in lymphocytes stimulated by phytohemagglutinin. These results indicate that jatrophone is a potent inhibitor of activation of lymphocytes, probably through inhibition of the protein kinase C pathway.

Keywords: Jatrophone; Natural killer activity; Lymphocyte proliferation; Protein kinase C; 12-*O*-Tetradecanoylphorbol 13-acetate; CD69; Interleukin-2

1. Introduction

Jatrophone is a naturally occurring diterpene isolated from the rhizome of the medicinal plant *Jatropha elliptica* (Euphorbiaceae). This diterpene exhibits a broad spectrum of biological actions. Thus, jatrophone causes long-lasting and graded relaxation of the rat uterus, guinea pig ileum, urinary bladder, dog ureters and it also inhibits the inotropism of paced rat left atrium (Calixto and Sant'Ana, 1987). Jatrophone also inhibits calcium chloride-mediated contraction of vascular and non-vascular smooth muscles (Calixto and Sant'Ana, 1990; Duarte et al., 1992). Recently, we have demonstrated that, like the protein kinase C inhibitors staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazinedihydrochloride (H7), jatrophone causes a dose-dependent relaxation in rings of rat portal vein contracted by the phorbol 12-acetate 13-myristate, noradrenaline, endothelin-1, KCl or caffeine (Silva et al., 1995). Furthermore, similar to staurosporine, jatrophone has a greater ability to inhibit contraction elicited by the phorbol 12-acetate 13-myristate, suggesting that its relaxant action may be associated with an interaction at the protein kinase C level.

Phorbol esters have been widely used as a tool for investigating the role of protein kinase C in controlling cell responses in different biological systems (Castagna et al., 1992), as in the case of the increased contractile respon-

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siveness of arteries from hypertensive rats (Braddshaw et al., 1993). Protein kinase C is a family of serine/threonine protein kinases playing a central role in the initiation of a cascade of biochemical events leading to lymphocyte activation (Berry and Nishizuka, 1990; Shenoy et al., 1993). Lymphocytes, depending on the stimulus, are able to proliferate or exert natural killer activity against certain tumor or virus-infected cells. This cytotoxicity occurs spontaneously after contact of large granular lymphocytes with their targets. It has been demonstrated that the expression of the surface protein CD69, which is indicative of natural killer cell activation (Borrego et al., 1993), and the cellular exocytosis of granular contents of lymphocytes (Ting et al., 1992) are mediated by stimulation of protein kinase C-dependent mechanisms. Among the biological agents that modulate natural killer activity is the plant diterpene 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a specific activator of protein kinase C (Procopio et al., 1989). In a previous study (Moraes et al., 1989), it was demonstrated that pre-treatment of lymphocytes with the phorbol ester TPA stimulated spontaneous natural killer activity associated with pronounced proliferation of the same lymphocyte population. Taken together these data led us to examine the effect of the naturally occurring diterpene jatrophone on the stimulation of natural killer activity and lymphocyte proliferation.

The results reported here show that jatrophone is a potent blocker of both proliferation and natural killer activity of lymphocytes stimulated by TPA. They also suggest that jatrophone can be a useful means of exploring the role of protein kinase C in different cellular activation processes.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells were obtained by the separation of heparinized blood from healthy volunteers by means of Ficoll-Hypaque density gradient centrifugation. Murine lymphocytes were obtained from normal BALB/c mice (INCa, RJ, Brazil). Spleens were lysed and forced through a steel mesh. After haemolysis with ammonium chloride buffer, cells were extensively washed by centrifugation and re-suspended in RPMI medium containing 5% fetal calf serum. Cells of the K_{562} cell line, derived from human erythroleukemia, were used in the proliferative assay and as targets in the natural killer assay.

2.2. Natural killer assay

The fraction containing peripheral blood mononuclear cells was re-suspended in RPMI medium and depleted of phagocytic mononuclear cells by adherence onto plastic flasks. After 1 h at 37°C, the non-adherent cells were re-suspended at a concentration of 5×10^6 cells/ml for use as effector cells. Before the assay, 10^6 target cells were labelled with 150 μCi $Na_2^{51}CrO_4$ for 90 min at 37°C. After two washes with RPMI containing 5% fetal calf serum, the cells were re-suspended at a concentration of 5×10^4 cells/ml in the same medium. Cytotoxicity was determined by using the ^{51}Cr release assay as described elsewhere (Moraes et al., 1989). Briefly, effector cells were used to give effector : target ratios of 100 : 1 (5×10^6 cells/ml : 5×10^4 cells/ml). After a 4 h incubation period, 100 μl of supernatant was collected and the radioactivity was assessed. The percentage of specific lysis was calculated from the following formula: {[(experimental release) − (spontaneous release)]/[(total release) − (spontaneous release)]} × 100.

2.3. Stimulation of natural killer activity by TPA

Peripheral blood mononuclear cells depleted of phagocytic mononuclear cells were pre-incubated in the absence or in the presence of 10 ng/ml TPA, alone or together with 1.1 μM jatrophone. After 1 h at 37°C, TPA and jatrophone were washed out from the cultures, and the non-adherent cells were re-suspended at a concentration of 5×10^6 cells/ml for use as effector cells to measure the natural killer activity.

2.4. Cell proliferation

Peripheral blood mononuclear cells (10^6 cells/ml) were stimulated by 5 μg/ml phytohemagglutinin or 100 ng/ml of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) plus 0.15 μM ionomycin for 96 h in flat-bottom 96-well microplates at 37°C in a 5% CO_2 atmosphere. Murine lymphocytes (10^6 cells/ml) were stimulated with 5 μg/ml concanavalin A. Proliferation was measured by [^{3}H]thymidine incorporation into cellular DNA; 0.5 μCi/well was added 6 h before the end of the culture period, which was 96 h for human and 72 h for murine lymphocytes. Cells were harvested and the radioactivity was assessed using a liquid scintillation counter. K_{562} cells (5×10^5 cells) were cultivated in 96-well microplates for 24 h and 48 h in the absence or in the presence of increasing concentrations of jatrophone. Proliferation was measured as above, except that the time for [^{3}H]thymidine incorporation was 1 h.

2.5. Additions

Jatrophone isolated from tubers of *Jatropha elliptica* as previously described (Duarte et al., 1992) was dissolved in ethanol to give a stock solution of 32 mM. Just before use, it was diluted to desired concentrations in RPMI containing 5% fetal calf serum to give less than 0.5% ethanol. Jatrophone aliquots were always added at the beginning of the natural killer activity assay or together with mitogen for the cell proliferation assay. Preliminary control experi-

ments demonstrated that jatrophone 'per se' did not affect the viability of the cells assessed by the trypan-blue exclusion method nor did it increase the spontaneous ^{51}Cr release from K_{562} cells.

2.6. Intracellular Ca^{2+} measurement

The increase in the free intracellular Ca^{2+} concentration was measured using fura-2 AM as a probe (Malgaroli et al., 1987). Peripheral blood mononuclear cells (5×10^6 cells/ml) were incubated in a medium containing 6 μM fura-2 AM and 2.5 mM probenecid for 1 h at 37°C. After this period, cells were washed twice with phosphate-buffered saline (PBS), re-suspended in PBS containing 1.6 mM $CaCl_2$, kept on ice and protected from light until used. Different concentrations of Jatrophone and 10 μg/ml phytohemagglutinin were added directly into fluorimeter cuvettes containing 5×10^5 cells/ml. During the assay, cells were kept at room temperature. Ca^{2+} concentration changes were monitored continuously using a fluorescence spectrophotometer (Hitachi F4500, Tokyo, Japan) set at 340 nm and 510 nm for excitation and emission, respectively. At the end of each recording, Triton X-100 was added to the cell suspension at a final concentration of 0.1% and the fluorescence measured was taken as maximal (100%). The minimal fluorescence (0%) was obtained by the addition of 50 mM EGTA pH 8.0 to the lysed cells.

2.7. Flow cytometric analysis for CD69 expression

Analysis for surface immunofluorescence was performed with a FACScan flow cytometer (Becton Dickinson, CA, USA). Cells after pre-incubation for 24 h with 10 ng/ml TPA or 300 IU IL-2 were washed 3 times with RPMI and re-suspended at 10^6 cells/ml. For measurement of immunofluorescence they were incubated with a saturating amount of anti-human Leu-23 (anti-CD69) for 30 min at 4°C, washed twice with chilled PBS, stained with goat anti-mouse fluorescein isothiocianate immunoglobulin (Ig FITC) for 30 min at 4°C and re-suspended in PBS containing 1% formaldehyde and 0.5% sodium azide. Non-specific binding was subtracted using appopriate controls.

2.8. Materials

RPMI 1640, EGTA, Triton X-100, probenecid (*p*-(dipropylsulfamoil benzoic acid), ionomycin, phytohemagglutinin (PHA), concanavalin A (ConA), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), Ficoll-Hypaque and [^{3}H]-thymidine (59 μCi/mmol) were purchased from Sigma Chemical Co. (St Louis, MO, USA); fura-2 AM was from Molecular Probes (Eugene USA); interleukin 2, Lymphocult-T-LF (IL-2), was from Biotest (Germany); anti-human Leu-23 antibody (anti-CD69) and goat anti-mouse Ig FITC were from Becton Dickinson (CA, USA); fetal calf serum was from Fazenda Pigue (Rio de Janeiro, Brazil); $Na_2^{51}CrO_4$ (252 μCi/mg Cr) was purchased from CNEN (São Paulo, Brazil). Stock solutions of TPA was prepared by dissolving 1 mg/ml in dimethyl sulfoxide (DMSO) and stored at −70°C.

2.9. Statistical analysis

Results are expressed as the means ± S.E.M. The concentration of jatrophone causing half maximal inhibition relative to control response (IC_{50}) is presented as the geometric mean accompanied by the 95% confidence limits and was determined from the individual concentration response curves by use of the least-squares method. Statistical significance of differences between groups was analysed by means of unpaired Student's *t*-test. Values of $P < 0.05$ were considered as indicative of significance.

3. Results

3.1. Effect of jatrophone on cellular proliferation

To investigate the effect of jatrophone on cellular activation, we added increasing concentrations to the culture medium containing human or murine lymphocytes and different mitogenic stimuli. Addition of jatrophone (0.02–0.32 μM) resulted in a dose-dependent and equipotent inhibition of human lymphocyte proliferation induced by 5 μg/ml phytohemagglutinin or by 100 ng/ml TPA plus 0.15 μM ionomicyn. The calculated mean IC_{50} values (and their 95% confidence limits) were 53.4 (42.6–65.3) nM and 48.4 (39.4–59.8) nM, respectively. To investigate whether this inhibition caused by jatrophone was directed against human lymphocytes, we also tested its effect on mouse lymphocytes. The results show that murine lymphocyte proliferation stimulated by 5 μg/ml concanavalin A, the mitogen usually used for these cells, was also blocked by jatrophone with an estimated mean IC_{50} value of 63.5 (51.2–76.5) nM. These data are summarized in Fig. 1.

The strong inhibitory effect of jatrophone on the proliferative response of lymphocytes of different origins could be due to a toxic effect on these cells. In order to check this possibility, human lymphocytes were pre-incubated with 0.32 μM jatrophone for 48 h at 37°C and 5% of CO_2 atmosphere. After this time period, cells were exhaustively washed with RPMI medium, re-suspended to 10^6 cells/ml and stimulated to proliferate with either phytohemagglutinin or with a combination of TPA plus ionomycin as described above. As shown in Fig. 2, human lymphocytes cultivated with jatrophone for 48 h proliferated to the same extent as control cells (vehicle-treated cells), after removal of the diterpene from the medium. It might be inferred from this result that jatrophone could be inactivated during the pre-incubation period. However, this was not the case

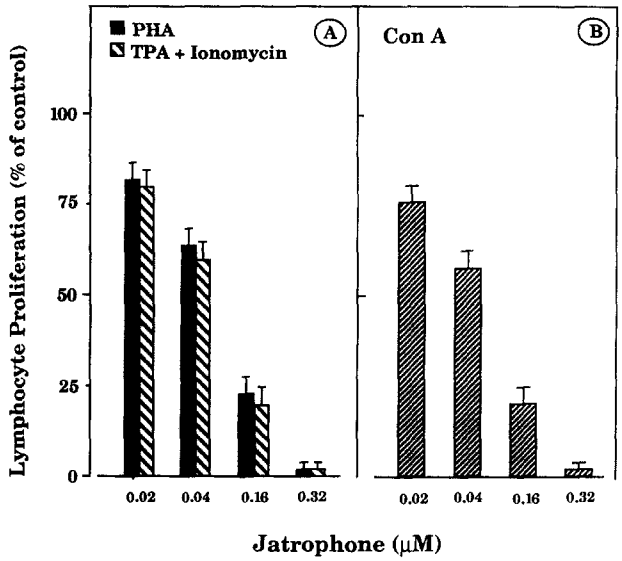

Fig. 1. The inhibitory effect of jatrophone on lymphocyte proliferation. (A) Human peripheral blood mononuclear cells (10^6 cells/ml) were incubated with 5 μg/ml phytohemagglutinin or 100 ng/ml TPA and 0.15 μM ionomycin for 96 h. The maximal level of incorporation taken as reference was 24196 cpm. (B) Mouse spleen lymphocytes (10^6 cells/ml) were incubated with 5 μg/ml concanavalin A for 72 h. The maximal level of incorporation taken as reference was 12917 cpm. Values are the means ± S.E.M. of five experiments.

as the addition of the supernatant from the pre-incubation period to proliferating cultures blocked lymphocyte proliferation (data not shown).

Table 1
Lack of inhibition of jatrophone on [^{3}H]thymidine incorporation by K_{562} cells

Conditions [a]	cpm [b]		
	6 h	24 h	48 h
Control	540 ± 20	2630 ± 184	5856 ± 404
+ jatrophone 0.08 μM	580 ± 15	2630 ± 253	5786 ± 556
+ jatrophone 0.16 μM	450 ± 18	2526 ± 208	5557 ± 457
+ jatrophone 0.32 μM	500 ± 35	2915 ± 316	6413 ± 615

[a] 100 μl aliquots of 5×10^5 K_{562} cells/ml were incubated in the absence or in the presence of increasing concentrations of jatrophone in a 96-well microplate, for 6, 24 or 48 h at 37°C and 5% CO_2. [b] 0.5 μCi [^{3}H]thymidine/well was added at the beginning, 18 h or 42 h of the culture period. Six hours later, cells were harvested and radioactivity was assessed. Each group represents the average ± S.E.M. of four determinations.

To further investigate the effect of jatrophone on a different cell population, the spontaneous growth of cells of the erythroleukemia-derived cell line K_{562} was measured in the presence of this diterpene. Table 1 shows that jatrophone did not inhibit the proliferation of the K_{562} cells.

3.2. Effect of jatrophone on stimulation of natural killer activity by TPA

Human lymphocytes were pre-incubated in the absence or in the presence of 10 ng/ml TPA for 1 h, alone or together with 1.1 μM jatrophone. After this time, TPA and

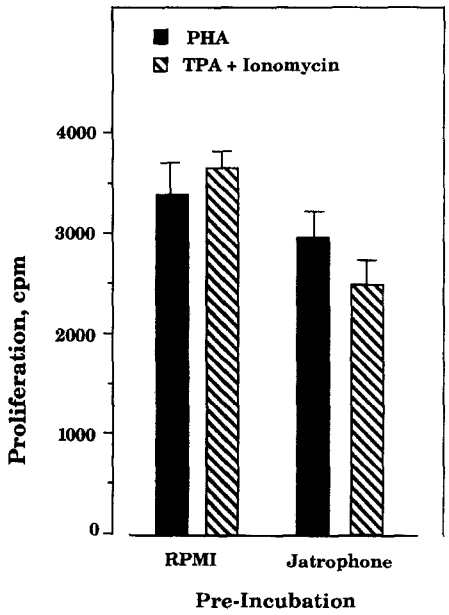

Fig. 2. Effect of pre-incubation of lymphocytes with jatrophone on proliferative response to phytohemagglutinin or TPA plus ionomycin. Peripheral blood mononuclear cells were cultivated for 48 h at 37°C and 5% CO_2 in the absence or in the presence of 0.32 μM jatrophone. After this time, cells were exhaustively washed, re-suspended to 10^6 cells/ml and incubated with phytohemagglutinin (black column) or TPA and ionomycin (hatched column) as described in Methods. Values are the means ± S.E.M. of four determinations.

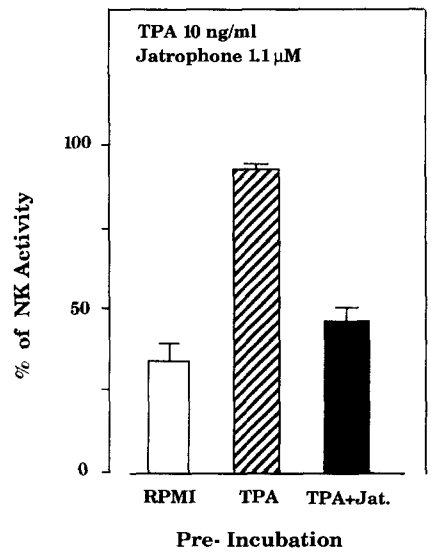

Fig. 3. Effect of jatrophone on TPA-stimulated natural killer activity. Phagocyte-depleted mononuclear cells (see Methods) were pre-incubated for 1 h at 37°C and 5% CO_2, in the presence of RPMI (blank column); 10 ng/ml TPA (hatched column) or 10 ng/ml TPA and 1.1 μM jatrophone (black column). After this time period, cells were washed several times with RPMI, re-suspended to 5×10^6 cells/ml and considered as effector cells. Natural killer activity was determined as described in Methods. Values are the means ± S.E.M. of four determinations.

jatrophone were washed out from the cultures, and the cells were used as effector cells to measure the natural killer activity. As shown in Fig. 3, the previous contact with TPA stimulated almost 3-fold the spontaneous natural killer activity against K_{562} cells. Addition of 1.1 μM jatrophone blocked the stimulation induced by TPA. Addition of increasing concentrations of jatrophone blocked both stimulated and spontaneous natural killer activity in a dose-dependent manner (data not shown).

3.3. Effect of jatrophone on intracellular Ca^{2+} levels in lymphocytes stimulated by phytohemagglutinin

Human lymphocytes were pre-loaded with fura-2 AM in the presence of probenecid, a blocker of organic anion transport systems and used to prevent Ca^{2+} flux into the extracellular medium (Di Virgilio et al., 1990). Cells were stimulated by 10 μg/ml phytohemagglutinin in the absence or in the presence of 0.08, 0.16 or 0.32 μM jatrophone. Fig. 4A shows the pattern of intracellular Ca^{2+} concentration change obtained after addition of 0.16 μM jatrophone (left arrow) before the addition of phytohemagglutinin to the fluorimeter cuvettes (right arrow). Identical results were obtained in the presence of PBS or 0.08 μM jatrophone (data not shown). In all cases the calculated rate assay value was 1.5. Fig. 4B shows that 0.32 μM jatrophone inhibited the increase in intracellular Ca^{2+} flux stimulated by phytohemagglutinin with a rate assay value of 0.3.

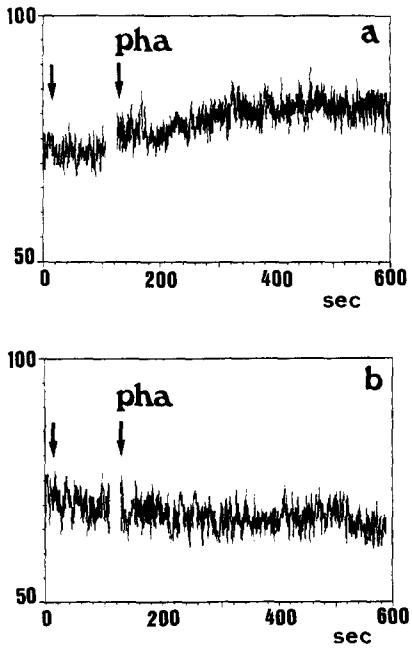

Fig. 4. Effect of jatrophone on $[Ca^{2+}]i$ in human lymphocytes stimulated by phytohemagglutinin. Lymphocytes were loaded with fura-2 AM and fluorescence was measured as described in Methods. In panel A, 0.16 μM jatrophone was added directly into fluorimeter cuvettes containing 5×10^5 cells/ml (left arrow). At 100 s, 10 μg/ml phytohemagglutinin was added (right arrow). In panel B, 0.32 μM jatrophone was added before phytohemagglutinin.

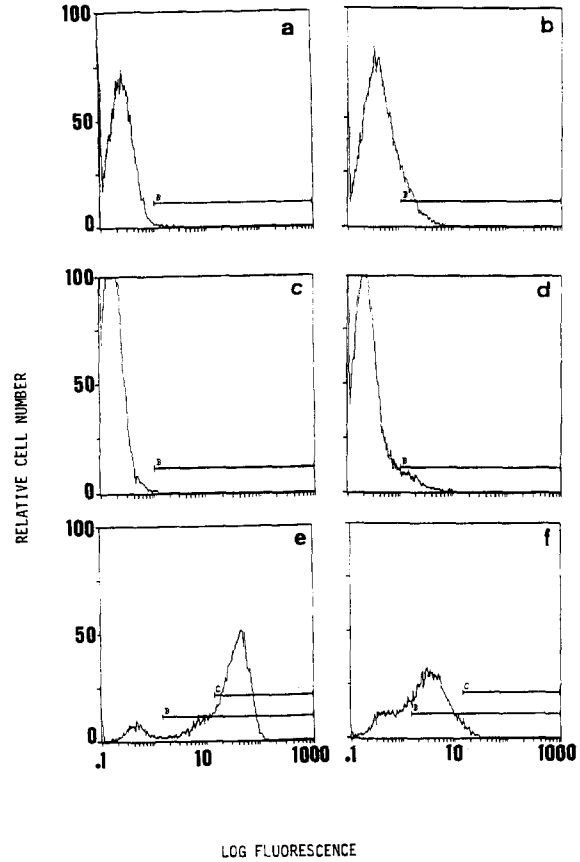

Fig. 5. Effect of jatrophone on the expression of CD69 stimulated by interleukin 2. Lymphocytes were activated for 24 h in the following conditions: (a) control resting cells; (b) interleukin 2 (300 IU); (c) 3.2 μM jatrophone; (d) interleukin 2 (300 IU) plus 3.2 μM jatrophone; (e) 10 ng/ml TPA; (f) 10 ng/ml TPA plus 3.2 μM jatrophone. After this time, cells were washed and used to measure the expression of CD69 in a FACScan flow cytometer as described in Methods. Histograms are representative of a typical experiment.

3.4. Effect of jatrophone on expression of CD69

Human lymphocytes (10^6 cells/ml) were stimulated with 10 ng/ml TPA or 300 IU interleukin 2 in the absence or in the presence of 3.2 μM jatrophone for 24 h. Fig. 5 shows that the expression of CD69 induced by TPA was completely inhibited by jatrophone. Jatrophone also partially affected the expression of CD69 induced by interleukin 2.

4. Discussion

Lymphocyte proliferation is an event triggered by the binding of effectors to the plasma membrane, generating second messengers such as the diacylglycerol that stimu-

lates protein kinase C activity (Nishizuka, 1986). Diterpene phorbol esters, due to their lipophilicity, cross the cell membrane and, like the endogenous diacylglycerol, activate protein kinase C (Castagna et al., 1992). Thus, addition of protein kinase C inhibitors blocks cellular proliferation stimulated by phorbol esters. This could be the explanation for the effect of jatrophone on TPA-induced lymphocyte proliferation. Furthermore, the IC_{50} values reported for jatrophone for inhibition of lymphocyte proliferation are in the same range as those obtained for this diterpene for blockade of phorbol-induced contractions of rat portal vein rings (Silva et al., 1995). Our results also show that jatrophone inhibited, with almost the same IC_{50} values, the proliferative response of lymphocytes to mitogens, phorbol ester or recombinant interleukin 2, but not the aberrant proliferation of K_{562} cells. The effect of jatrophone was also investigated in murine lymphocytes. The results indicate that this diterpene can act on the proliferative pathway of both types of lymphocytes, suggesting blockade of a common step in the proliferative response such as the protein kinase C pathway.

These data led us to investigate the effect of jatrophone on the natural killer activity of lymphocytes that is dependent on protein kinase C activity and which can also be stimulated by phorbol ester (Procopio et al., 1989). Previous results (Moraes et al., 1989) and data from the literature (Shenoy et al., 1993) have demonstrated that previous treatment of lymphocytes with the phorbol ester TPA markedly increases the spontaneous natural killer activity. Our results demonstrated that this stimulation of natural killer activity induced by the specific activator of protein kinase C was completely blocked by jatrophone, indicating a blockade via protein kinase C pathway.

Recently, it was reported that in preparations of rat portal vein precontracted with the phorbol ester, jatrophone, H7 and staurosporine caused graded relaxations. Jatrophone also inhibited in a graded manner caffeine-induced contractions. These observations indicate that the vasorelaxant action of jatrophone is similar to that reported for inhibitors of protein kinase C activity, leading to the conclusion that jatrophone is acting through inhibition of protein kinase C-dependent mechanisms. In parallel, it was observed that jatrophone, depending on the concentration, could also inhibit the Ca^{2+} flux (Silva et al., 1995). Our results agree with these previous observations. In the present case, jatrophone concentrations that block 75% of the proliferative response did not affect the increase in intracellular Ca^{2+} flux of phytohemagglutinin-stimulated lymphocytes. Furthermore, jatrophone in concentrations that completely blocked proliferation impaired the stimulation of natural killer activity and expression of CD69 of lymphocytes induced by TPA, affecting only partially the expression of CD69 stimulated by interleukin 2. These results are in agreement with data showing that the expression of CD69 stimulated by interleukin 2 is not affected by staurosporine (Borrego et al., 1993). The effect of high doses of jatrophone on Ca^{2+} flux might be explained as being a consequence of the blockade of the activation of the protein kinase C pathway, that would precede the release of Ca^{2+} from intracellular stores (Alonso-Torre and Trautmann, 1995).

These findings strongly suggest that the inhibitory effect of the naturally occurring diterpene jatrophone is associated with an effect on the protein kinase C pathway. Jatrophone's in vivo effects are now under investigation.

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