Phenylarsine oxide and phorbol myristate acetate inhibit the CD3-induced rise of cytosolic Ca^{2+} in Jurkat cells by refilling internal Ca^{2+} stores

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Phenylarsine oxide (PAO), an inhibitor of tyrosine phosphatases, has been found to inhibit the early elevation in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), related to the CD3 activation pathway in Jurkat T cells. This inhibition was dose-dependent, consistent with previously reported effects of PAO on tyrosine phosphatases, and reversed by dimercaptopropanol. By contrast, okadaic acid, an inhibitor of serine/threonine phosphatases, had no effect on CD3-induced Ca^{2+} flux. PAO was compared with phorbol 12-myristate 13-acetate (PMA), which caused a similar, although less potent, inhibition as previously described. The two reagents produced additive inhibition of the CD3-induced $[\text{Ca}^{2+}]_i$ rise, but did not affect thapsigargin- or ionomycin-driven Ca^{2+} flux in

Jurkat cells. PAO and PMA prevented cells from complete depletion of intracellular Ca^{2+} stores by an anti-CD3 monoclonal antibody (mAb) and restored, at least partially, the ionomycin-sensitive pool, when added after anti-CD3 mAb. Moreover, the CD3-induced inhibition of phosphatidylserine synthesis, due to depletion of internal Ca^{2+} stores, is reversed by PAO and PMA. Anti-phosphotyrosine immunoblot analysis show that these effects cannot be accounted for by an inhibition of CD3-induced tyrosine phosphorylations. We propose that PAO and, to a lesser extent, PMA allow the refilling of internal compartments by Ca^{2+} , which consequently abrogates a capacitative entry of external Ca^{2+} .

INTRODUCTION

Activation of T lymphocytes by mitogenic lectins or monoclonal antibodies (mAb) trigger an early increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which appears of most importance for stimulation of T cells, because depletion of extracellular Ca^{2+} abrogates the activation process, and conversely Ca^{2+} ionophores can be mitogenic [1–6]. In Indo-1-loaded T cells, the CD3-induced changes in $[\text{Ca}^{2+}]_i$ comprise a large transient peak and a lower sustained plateau [7]. The first phase is attributed to release from internal stores, and the second, which depends on extracellular Ca^{2+} concentration, results from a transmembrane uptake due to an increased rate of influx, a decreased rate of efflux, or both. The sensitivity of this uptake to pharmacological drugs does not match any characterized Ca^{2+} channel, and it has often been proposed that Ca^{2+} uptake and release are linked, whether they are operated by related mediators [8], or according to a capacitative model [9]. This last hypothesis was reinforced especially as specific inhibitors of endoplasmic-reticulum Ca^{2+} -ATPase are able to trigger Ca^{2+} entry in many cell types [10–12], and to activate T cells [13].

In Jurkat T cells phorbol 12-myristate 13-acetate (PMA) and other protein kinase C activators have been reported to impair CD3-mediated Ca^{2+} flux [14] by some unknown mechanism. Because PMA triggers protein phosphorylations which can also be regulated by phosphatases, we looked at the effects of phosphatase inhibitors on the Ca^{2+} response of Jurkat cells to anti-CD3 mAb. We found that phenylarsine oxide (PAO), but not okadaic acid, had a similar but more potent effect than PMA. In both cases the inhibition could be a consequence of a reversal of the CD3-induced depletion of internal Ca^{2+} stores.

MATERIALS AND METHODS

Cells

The human T cell line Jurkat was kindly supplied by Dr. A. M. Schmitt-Verhulst (Centre d'Immunologie, Marseille-Luminy, France). Cells were cloned by limiting dilution. Clone D was selected on the base of its interleukin-2 production when activated with phytohaemagglutinin and the phorbol ester PMA. Cells were cultured in RPMI 1640 (Seromed, Lille, France) supplemented with 5% (v/v) fetal-calf serum, 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 0.1 μM β -mercaptoethanol.

Reagents and antibodies

Ionomycin, PMA, thapsigargin, EGTA, Hepes, leupeptin, phenylmethanesulphonyl fluoride, aprotinin, Nonidet P-40 and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). PAO was from Aldrich (St. Quentin Fallavier, France) and okadaic acid from Boehringer Mannheim (Mannheim, Germany). [^3H]Serine (0.37–1.1 TBq/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). All reagents for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.) and Immobilon-P transfer membrane was from Millipore (Bedford, MA, U.S.A.).

Rabbit anti-mouse (RAM) Ig was from Dako (Dakopatts, Copenhagen, Denmark) and anti-phosphotyrosine mAb 4G10 was from UBI (Lake Placid, NY, U.S.A.). The anti-CD3 mAb X35 was kindly provided by Dr. D. Bourel (Lille, France).

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; PAO, phenylarsine oxide; PMA, phorbol 12-myristate 13-acetate; PtdSer, phosphatidylserine; InsP_3 , inositol 1,4,5-trisphosphate; RAM, rabbit anti-mouse Ig; mAb, monoclonal antibody; TCR, T-cell receptor.

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Phosphatidylserine (PtdSer) synthesis

Jurkat cells (2×10^6) were maintained in 0.5 ml of a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 2.5 mM glucose, 20 mM Hepes, 0.1 % BSA, 1 mM MgCl_2 and 1 mM CaCl_2 at 37 °C in the presence of 4 $\mu\text{Ci/ml}$ [^3H]serine (10–30 Ci/mmol) (Amersham) and effectors (see concentrations in the Figure legends). After an incubation period ranging from 0 to 2 h, the cells were rapidly sedimented in an Eppendorf centrifuge, the supernatants were discarded, and the cell phospholipids were extracted with chloroform/methanol. The lipid extracts were analysed by t.l.c. in a solvent system composed of chloroform/methanol/acetic acid/water (25:5:4:1, by vol.). Authentic phospholipid standards (Sigma) were run in parallel and detected with I_2 vapour. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyser (Berthold).

Measurements of changes in Ca^{2+}

The assay of $[\text{Ca}^{2+}]_i$ was performed by using Indo-1 (Sigma) [15]. Cells ($5 \times 10^6/\text{ml}$) were loaded with 5 μM Indo-1 at 37 °C in the dark for 1 h, then washed and resuspended in Hepes-buffered saline. Analyses were performed on a dual-laser fluorescence-activated cell sorter (FACStar plus; Becton Dickinson). The fluorescence intensities at 480 nm corresponding to the free Indo-1 concentration and at 400 nm corresponding to the complex Ca^{2+} -Indo-1 were measured. The 400 nm/480 nm fluorescence ratio, in arbitrary units, gives an estimate of $[\text{Ca}^{2+}]_i$, independent of the cell size and the intracellular Indo-1 concentration. Side and forward scatter of blue laser light were used to gate out damaged cells and debris. The cell flow rate was about 1000/s. Stability of the baseline was checked during 1 min, then effectors were added, and the mean 400 nm/480 nm ratio of 3000 cells was noted every 30 s.

Anti-phosphotyrosine immunoblot analysis

Jurkat cells were washed twice with culture medium. Cells were suspended at 5×10^6 cells/ml in RPMI containing 5 % foetal-calf serum and 25 mM Hepes, pH 7.5, and then incubated with anti-CD3 mAb X35 (2 $\mu\text{g/ml}$) for 5 min at 37 °C, alone or in the presence of PAO (2 μM), PMA (0.1 μM) or both. Stimulations were terminated by addition of 500 μl of ice-cold stop buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4). Cells were spun down and pellets were resuspended in 60 μl of lysis buffer (stop buffer with 1 % Nonidet P40, 10 $\mu\text{g/ml}$ leupeptin, 1 mM phenylmethanesulphonyl fluoride, 1000 units/ml aprotinin) and incubated on ice for 30 min. Whole cell lysates were centrifuged at 15000 g for 10 min, and cellular extracts were analysed by SDS/PAGE on a 12 %-acrylamide gel, followed by transfer to an Immobilon-P membrane (Millipore). The membranes were saturated for 16 h at 4 °C in Buffer A (50 mM Tris/HCl, 150 mM NaCl, 5 % BSA, pH 7.5), then incubated in the same buffer with 1 $\mu\text{g/ml}$ anti-phosphotyrosine mAb 4G10 (UBI) for 2 h at 23 °C. The membranes were washed four times in Buffer B (50 mM Tris/HCl, 150 mM NaCl, 0.4 % BSA, 0.5 % Nonidet P40, pH 7.5), incubated for 1 h at 23 °C with RAM (1:1000) in Buffer A and washed four times in Buffer B. The membranes were finally incubated with ^{125}I -Protein A (500000 c.p.m./ml) for 1 h at 23 °C in Buffer A, washed four times in Buffer B and subjected to autoradiography.

RESULTS

PAO inhibits CD3-induced Ca^{2+} flux

PMA has already been shown to inhibit Ca^{2+} flux in Jurkat T cells [14]; we confirmed that PMA decreased by 60–70 % the sustained $[\text{Ca}^{2+}]_i$ elevation following CD3 stimulation of Jurkat T cells, but was less effective on the release from internal stores (results not shown). To investigate whether this impairment was due to protein phosphorylation, we studied the effects of protein phosphatase inhibitors. We found that the CD3-induced $[\text{Ca}^{2+}]_i$ increase was considerably lowered after a few minutes' exposure to PAO. This agent reacts with vicinal disulphide groups to form stable cyclic complexes, and it has been described as an inhibitor of protein tyrosine phosphatases [16]. The effect of PAO was dose-dependent, with an EC_{50} of about 0.5 μM (Figure 1a), and was reversed by 80 μM dimercaptopropanol, a bifunctional thiol-reducing agent which was shown to reverse PAO effects [17]. On the contrary, 10 μM okadaic acid, an inhibitor of serine/threonine protein phosphatases 1 and 2A [18], had little effect on the CD3-triggered Ca^{2+} flux (Figure 1b). High concentrations of PAO (from 6 μM) resulted in a slow elevation of $[\text{Ca}^{2+}]_i$ which would interfere with CD3-dependent flux, and therefore were not further studied.

When external Ca^{2+} was chelated by EGTA, the CD3-induced transient $[\text{Ca}^{2+}]_i$ elevation, due to the mobilization of intracellular stores, was still inhibited by PAO, but to a lesser extent than the external- Ca^{2+} -dependent plateau (Figure 1c). Thereafter the concentration of PAO was limited to 6 μM , because of the appearance of a secondary rise of Ca^{2+} level in unstimulated cells.

To check whether the effect of PAO required a preincubation, or could be observed after activation of the CD3 pathway, Jurkat cells were first stimulated for 5 min, to reach the beginning of the sustained external- Ca^{2+} -dependent $[\text{Ca}^{2+}]_i$ increase, and then PAO was added. Figure 1(d) demonstrates that PAO, like PMA (results not shown), reversed the CD3-primed Ca^{2+} influx and returned $[\text{Ca}^{2+}]_i$ to near the resting level.

These data indicate that the effects of PAO resemble those of PMA in inhibiting essentially the sustained phase of $[\text{Ca}^{2+}]_i$ elevation in activated cells. The additivity of the two effectors was analysed. PMA-induced inhibition was found to be optimal, but not total, at 0.1 μM . Figure 2 shows that a sub-optimal dose of PAO could additionally impair both transient and sustained phases of the CD3-induced $[\text{Ca}^{2+}]_i$ rise in Jurkat cells.

The inhibition by PAO and PMA of the $[\text{Ca}^{2+}]_i$ increase could be due to an decrease of the CD3-triggered Ca^{2+} entry or to an improved Ca^{2+} extrusion by more efficient Ca^{2+} pumps. To distinguish between these two possibilities, we analysed the effects of PAO and PMA on $[\text{Ca}^{2+}]_i$ elevation driven by ionomycin and thapsigargin.

PAO and PMA poorly affect ionomycin- or thapsigargin-induced $[\text{Ca}^{2+}]_i$ variations

Ionomycin, as a Ca^{2+} ionophore, increases the permeability of lipid membranes to Ca^{2+} ions, which allows an elevation of $[\text{Ca}^{2+}]_i$ due to both a release from internal stores and an influx from external medium. When it is added at 0.1 μM to Jurkat cells, the variations of $[\text{Ca}^{2+}]_i$ are similar to, although more rapid than, that observed after CD3 stimulation. On the other hand, thapsigargin, by specifically blocking the reticulum Ca^{2+} -ATPase, results in an uncompensated leakage of the intracellular pool of Ca^{2+} ; by some unknown mechanism, emptying internal stores triggers an influx of external Ca^{2+} through the plasma membrane. At 0.1 μM , with Jurkat cells, thapsigargin and ionomycin roughly mimic the CD3-dependent sustained plateau of $[\text{Ca}^{2+}]_i$. Neither

Figure 1 Effects of PAO on CD3-induced Ca^{2+} flux in Jurkat cells

(a) Anti-CD3 mAb X35 ($2 \mu\text{g}/\text{ml}$) was added to the cell suspension at 0 min in the presence of external Ca^{2+} (1 mM) and increasing concentrations of PAO ($\square$, 0; $\blacksquare$, $0.02 \mu\text{M}$; $\circ$, $0.06 \mu\text{M}$; $\bullet$, $0.2 \mu\text{M}$; $\triangle$, $0.6 \mu\text{M}$; $\blacktriangle$, $2 \mu\text{M}$; ∇ , $6 \mu\text{M}$). Analysis of Ca^{2+} flux was performed as described in the Materials and methods section. (b) Anti-CD3 mAb ($2 \mu\text{g}/\text{ml}$) was added to the cell suspension as above in the presence of medium ($\square$), PAO ($2 \mu\text{M}$) ($\bullet$), PAO ($2 \mu\text{M}$) and dimercaptopropanol (DMP; $80 \mu\text{M}$) ($\blacksquare$), or okadaic acid (OA; $10 \mu\text{M}$) ($\circ$). Cells were preincubated for 15 min at 23°C with okadaic acid before addition of anti-CD3 mAb. (c) Conditions and symbols were as in (a), except that external Ca^{2+} was chelated by EGTA (2 mM) before addition of anti-CD3 mAb. (d) Cells were stimulated for 5 min with anti-CD3 mAb ($2 \mu\text{g}/\text{ml}$) in the presence of external Ca^{2+} (1 mM). Then increasing concentrations of PAO were added to the cell suspension ($\triangle$, 0; $\square$, $0.02 \mu\text{M}$; $\blacksquare$, $0.06 \mu\text{M}$; $\circ$, $0.2 \mu\text{M}$; $\bullet$, $0.6 \mu\text{M}$; $\triangle$, $2 \mu\text{M}$) and analysis was continued.

Figure 2 Additive effects of PAO and PMA on CD3-induced Ca^{2+} flux in Jurkat cells

Anti-CD3 mAb X35 ($2 \mu\text{g}/\text{ml}$) was added to the cell suspension at 0 min in the presence of medium ($\square$), $0.1 \mu\text{M}$ PMA ($\circ$), $0.6 \mu\text{M}$ PAO ($\blacksquare$) or $0.1 \mu\text{M}$ PMA and $0.6 \mu\text{M}$ PAO ($\bullet$). Analysis of Ca^{2+} flux was performed as described in the Materials and methods section.

PMA nor PAO, nor a combination of both effectors, had a significant effect on those two pharmacologically modified Ca^{2+} kinetics (Figures 3a and 3c). They were no longer able to modify the plateau values when added after the effectors. Furthermore, thapsigargin and ionomycin remained able to reverse the inhibitions by PMA or PAO of the CD3-induced sustained $[\text{Ca}^{2+}]_i$ elevation (Figures 3b and 3d).

It thus seemed unlikely that PAO- and PMA-induced inhibition could be due either to improved extrusion, or to a blockade of the putative signal transmission between Ca^{2+} stores and plasma membrane. According to the capacitative model, an alternative

hypothesis was that PAO and PMA abrogated the CD3-induced Ca^{2+} entry by refilling internal stores, which otherwise remained depleted after stimulation of cells. The status of Ca^{2+} stores was thus further examined.

An ionomycin-releasable internal pool of Ca^{2+} is restored by PAO and PMA

Because of the lack of effect of PAO and PMA on ionomycin-dependent Ca^{2+} movements, we can assume that, in the absence of external Ca^{2+} , the ionomycin-induced transient peak of $[\text{Ca}^{2+}]_i$ is representative of the amount of Ca^{2+} stored in the cells. In the presence of EGTA (2 mM) to chelate external Ca^{2+} (1 mM), a first exposure to anti-CD3 mAb or to thapsigargin prevented Jurkat cells from responding to ionomycin. In the presence of $2 \mu\text{M}$ PAO or $0.1 \mu\text{M}$ PMA, however, a partial response to ionomycin was observed after CD3 treatment, but not to thapsigargin treatment (Figure 4a). In another experiment, cells were stimulated in the presence of 1 mM external Ca^{2+} , and PAO, PMA or ethanol (control) was added after 5 min. External Ca^{2+} was then chelated by EGTA, and the ionomycin-releasable pool was measured. Figure 4(b) shows that in those conditions, too, PAO and to a lesser extent PMA partially restored the ionomycin-induced Ca^{2+} release. On the contrary, ionomycin remained ineffective after an exposure to thapsigargin, in the absence or presence of PAO.

PAO and PMA reverse the CD3-induced inhibition of PtdSer synthesis

In Jurkat cells [19], as in other cells [20], the depletion of intracellular Ca^{2+} pools resulted in an inhibition of PtdSer synthesis. It was thus relevant to check whether PAO and PMA, which seemed to impair the sustained depletion of internal stores

Figure 3 Effects of PAO and PMA on thapsigargin- or ionomycin-induced Ca^{2+} flux in Jurkat cells

(a) Cells were treated at 0 min with 0.1 μM thapsigargin (Tg) in the presence of 1 mM Ca^{2+} and medium ($\square$), 0.1 μM PMA ($\circ$), 0.6 μM PAO ($\blacksquare$) or 0.1 μM PMA and 0.6 μM PAO ($\bullet$). (b) Cell suspensions treated at 0 min with anti-CD3 mAb X35 (2 $\mu\text{g}/\text{ml}$) ($\square$ and $\blacksquare$) or 0.1 μM thapsigargin ($\circ$ and $\bullet$) were analysed as described in the Materials and methods section. At $t = 4$ min, 2 μM PAO ($\bullet$ and $\blacksquare$) or 0.1 μM PMA ($\circ$ and $\square$) was added to the cell suspension and analysis was continued as above. At $t = 7$ min, 0.1 μM thapsigargin ($\square$ and $\blacksquare$) was added to the X35-treated cells and analysis was continued again. (c) The conditions were identical with those in (a) except that 0.1 μM ionomycin (Iono) was used in place of thapsigargin. (d) All conditions were as in (b), except that 0.1 μM ionomycin was used in place of thapsigargin.

Figure 4 Restoration by PAO and PMA of an ionomycin-releasable internal pool of Ca^{2+} in Jurkat cells

(a) At 0 min, cells were treated with anti-CD3 mAb X35 (2 $\mu\text{g}/\text{ml}$) ($\square$, $\circ$, $\blacksquare$) in the presence of medium ($\square$), 0.1 μM PMA ($\circ$) or 2 μM PAO ($\blacksquare$), or with 0.1 μM thapsigargin (Tg) in the presence of 0.1 μM PMA and 2 μM PAO ($\bullet$). External Ca^{2+} was chelated with EGTA (2 mM) before stimulation. At $t = 5$ min, ionomycin (0.1 μM) was added to the cell suspension and analysis of Ca^{2+} flux was continued. (b) Cells were treated at 0 min with anti-CD3 mAb (2 $\mu\text{g}/\text{ml}$) ($\square$, $\circ$, $\blacksquare$) or with 0.1 μM thapsigargin ($\bullet$) in the presence of 1 mM external Ca^{2+} . The following reagents were then added to the cell suspension: at $t = 5$ min, 0.1 μM PMA ($\circ$), 2 μM PAO ($\blacksquare$), 0.1 μM PMA and 2 μM PAO ($\bullet$) or control ($\square$); at $t = 7$ min, EGTA (2 mM) was added to chelate external Ca^{2+} ; at $t = 9$ min, ionomycin (0.1 μM) was added.

after CD3 treatment, could also reverse the subsequent inhibition of PtdSer synthesis. Cells were treated for 1 h with 2 $\mu\text{g}/\text{ml}$ anti-CD3 mAb, resulting in a decreased incorporation of [^3H]serine into PtdSer (untreated cells 560 ± 30 c.p.m.; CD3-treated cells 410 ± 25 c.p.m.) (Figure 5, 0 min). Then 2 μM PAO, 10 nM PMA or vehicle (ethanol) was added to cells, and PtdSer synthesis was monitored during 30 min. As shown in Figure 5, after PMA treatment PtdSer synthesis was significantly increased during the first 10 min and then returned to the control value. In contrast, PAO completely reversed the CD3-induced inhibition, and after

30 min the PtdSer synthesis was restored to the level of untreated cells.

PAO and PMA increase the CD3-induced tyrosine phosphorylation of cellular proteins

Stimulation of Jurkat T cells through the CD3/TCR (T-cell receptor) complex induces rapidly the phosphorylation on tyrosine residues of several cellular proteins [21–23], particularly PLC γ , which, by generating InsP_3 , is supposed to mediate the

Figure 5 Effects of PAO and PMA on the CD3-induced inhibition of PtdSer synthesis

Jurkat cells (2×10^6), in the presence of [^3H]serine, were stimulated or not (○) for 1 h at 37 °C with anti-CD3 mAb X35 (2 $\mu\text{g}/\text{ml}$). They were then treated with 2 μM PAO (●), 10 nM PMA (■) or vehicle (ethanol) (□) for the indicated times at 37 °C. Phospholipids were extracted and analysed as described in the Materials and methods section, and results (c.p.m.) are mean values $\pm$ S.D. ($n = 4$).

Figure 6 Effects of PAO and PMA on CD3-induced tyrosine phosphorylation of cellular proteins in Jurkat cells

Jurkat cells (2.5×10^6) were stimulated for 5 min at 37 °C without (lanes 1, 6, 7, 8) or with anti-CD3 mAb X35 (2 $\mu\text{g}/\text{ml}$) (lanes 2, 3, 4, 5) in the presence of PAO (2 μM) (lanes 2, 3, 6, 8) or PMA (0.1 μM). Anti-phosphotyrosine immunoblot analysis of whole cell lysates were performed as described in the Materials and methods section, and the membrane was autoradiographed. C, control.

release of Ca^{2+} from internal stores [22,24]. Therefore we checked whether the inhibitory effect of PAO and PMA on the CD3-induced Ca^{2+} flux was not due to inhibition of CD3-induced

tyrosine phosphorylations. Cells were exposed to 2 μM PAO, 0.1 μM PMA, both, or 1 % ethanol (vehicle) for 5 min at 37 °C, and whole cell lysates were subjected to anti-phosphotyrosine immunoblot analysis. As shown in Figure 6, PAO or PMA induced the phosphorylation on tyrosine residues of cellular proteins. These effects were consistent with previous reports in human T cells as well as in different cell types [16,25,26]. PAO, and in a lesser extent PMA, increased the CD3-induced tyrosine phosphorylations of cellular substrates. In addition, the combination of both reagents has an additive effect on the level of phosphotyrosine induced by CD3 (Figure 6, lane 5).

DISCUSSION

The elevation of $[\text{Ca}^{2+}]_i$ is one of the major early events that are thought to initiate the CD3/TCR-mediated activation cascade in T lymphocytes [2,27]. The $[\text{Ca}^{2+}]_i$ level in stimulated cells results from the mobilization of intracellular pools and from a modified balance between Ca^{2+} extrusion and influx. The mechanisms by which the membrane permeability to Ca^{2+} is increased by mitogenic antibodies and lectins are not clearly elucidated; the recent finding that thapsigargin, an inhibitor of the reticulum Ca^{2+} -ATPase, was able to trigger an entry of Ca^{2+} from extracellular medium, gave rise to a previously hypothesized model which linked the Ca^{2+} release and influx [28,29]. According to this capacitative model, emptying the internal stores would mediate, via some unknown pathway, an influx of external Ca^{2+} . The impairment of the CD3-related sustained elevation of $[\text{Ca}^{2+}]_i$ could thus be due to: (1) inhibition of the recognition of the CD3 complex by the mAb; (2) blockade of very early transduction signals; (3) the improvement of Calcium extrusion; or (4) inhibition of Ca^{2+} influx. As regards PMA- and PAO-induced inhibition, the first hypothesis can be rejected, because those two compounds did not modify the binding of fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb, at least during the first 10 min (results not shown). PMA is known to down-regulate the CD3/TCR expression on T lymphocytes, but the exposure must last 30 min to give a detectable effect. PAO had no effect, even after several hours. Besides, both reagents are able to reverse the sustained increase of $[\text{Ca}^{2+}]_i$, even if they are added after the achievement of the plateau phase. The observed inhibition of the CD3-dependent $[\text{Ca}^{2+}]_i$ rise was not due to an increase in Ca^{2+} extrusion by plasma-membrane Ca^{2+} -ATPase because in such a case ionomycin- and thapsigargin-induced $[\text{Ca}^{2+}]_i$ should have been affected, too. We can therefore presume that PMA and PAO specifically inhibit the CD3/TCR-triggered signal transduction. The release of Ca^{2+} from internal stores is supposed to be mediated by InsP_3 , generated after phosphorylation of $\text{PLC}\gamma$ on tyrosine residues by a kinase associated with the CD3/TCR complex, which may be $\text{p}59^{\text{lyn}}$ protein [22,24]. It seems unlikely that this mechanism could be directly affected by either PMA, which enhances in T cells serine/threonine and tyrosine phosphorylations via protein kinase C [30,31], or PAO, which increases tyrosine phosphorylations as a result of inhibition of tyrosine phosphatases [16,25]. This latter effect of PAO on tyrosine phosphorylation can also be accounted for by activation of a tyrosine kinase activity (or a combination of both mechanisms) [26]. However, our results favour the inhibition of tyrosine phosphatase activity (see Figure 6), as PAO (and PMA) enhance the CD3-induced tyrosine phosphorylations.

On the other hand, the sustained extracellular- $[\text{Ca}^{2+}]_i$ -dependent elevation is much more inhibited than the initial Ca^{2+} release. Recent work has brought several pieces of evidence that in many cell types a Ca^{2+} entry was secondary to the depletion of internal stores [11,32], and we demonstrate that CD3-activated

Jurkat cells appear to follow that capacitative model [33]. Because PMA and PAO do not seriously modify the thapsigargin-induced Ca^{2+} intake, they presumably do not interfere with the signalling pathway from empty stores to plasma membrane. Furthermore, it is unlikely that the effects of PAO are the results of uncoupling mitochondrial oxidation, because the depletion of intracellular ATP would need prolonged action of the reagent, and would result in an increase of the intracellular level of Ca^{2+} , due to the inhibition of ATPase. Furthermore, it would have the same action on the thapsigargin-induced Ca^{2+} increase. We established that PMA and PAO prevent cells from sustained depletion of internal stores, by measuring independently the ionomycin-releasable Ca^{2+} pools, and the synthesis of PtdSer, which we have shown to be strictly dependent on the Ca^{2+} level within the endoplasmic reticulum [19]. This finding consistently supports the capacitative model for Ca^{2+} influx, in which the restoration of internal Ca^{2+} pools abrogates the external- Ca^{2+} intake. This restoration cannot be observed with thapsigargin, which directly blocks the reticulum Ca^{2+} pump, nor with ionomycin, which dramatically decreases the membrane permeability to Ca^{2+} . Whether it occurs by closing the InsP_3 -sensitive Ca^{2+} channels or by accelerating the internal calcium pump cannot yet be decided and will need further investigations.

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