Induction of tyrosine phosphorylation and T-cell activation by vanadate peroxide, an inhibitor of protein tyrosine phosphatases

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Rapid tyrosine phosphorylation of key cellular proteins is a crucial event in the transduction of activation signals to T-lymphocytes. The regulatory role of protein tyrosine phosphatases (PTPases) in this process was explored by studying the effects of a powerful PTPase inhibitor, vanadate peroxide (pervanadate), on the activation cascade of Jurkat human leukaemic T-cells. Pervanadate induced activation of the tyrosine kinases lck and fyn (4- and 3-fold respectively) and a dramatic increase in tyrosine phosphorylation of cellular proteins, notably phospholipase C γ 1. After this event, we observed a rise in intracellular Ca^{2+} concentration, corresponding to an influx. This effect required surface expression of the CD45 PTPase and was not observed in CD45-deficient variants of Jurkat cells. In the CD45-negative variant, the effect of pervanadate on tyrosine phosphorylation was globally decreased and some phosphoryl-

ated substrates were specifically missing. Pervanadate also stimulated transcription of the *c-fos* gene and accumulation of its mRNA as well as several other hallmarks of T-lymphocyte activation such as surface expression of the CD69 antigen and the interleukin 2 receptor α -chain (CD25). Pervanadate synergized with signals delivered by T-cell antigen receptor engagement or by a phorbol ester to induce interleukin 2 production. Pervanadate activated NF- κ B, as shown by an increase in DNA-binding activity of this transcription factor. We thus conclude that PTPases play a crucial role in the negative regulation of signal transduction culminating in T-lymphocyte activation. Moreover, induction of tyrosine phosphorylation appears sufficient *per se* to initiate a complete activation programme.

INTRODUCTION

Antigen recognition by T-lymphocytes through the T-cell antigen receptor, TcR–CD3, is a central event in the initiation of an immune response. This multimeric complex consists of an antigen-binding clonotypic α/β heterodimer (TcR) non-covalently associated with the invariant chains of the CD3 complex γ , δ and ϵ and the disulphide-linked homodimer ζ – ζ [1,2]. Stimulation of the TcR–CD3 complex by antigen or agonistic monoclonal antibodies (mAbs) raised against TcR or CD3 activates a signal-transduction cascade. The earliest event in this cascade is a stimulation of tyrosine phosphorylation of several cellular proteins [3,4]. Tyrosine kinase (PTK) inhibitors have been shown to block early as well as late events resulting from TcR–CD3 triggering [5,6], suggesting that tyrosine phosphorylation is absolutely required for all downstream events in the T-cell response to antigen. Moreover, stimulation of the T-cell receptor leads to an increase in tyrosine phosphorylation of phospholipase C γ 1 (PLC γ 1) which is likely to induce an augmentation of its catalytic activity [7,8] and the subsequent generation of the second messengers inositol trisphosphate (IP $_3$) and diacylglycerol [9]. As the TcR–CD3 does not possess intrinsic kinase activity, it is probably associated with independent PTK(s). Candidates for this role are fyn and lck, two members of the src tyrosine kinase family [10,11]. Recently, fyn has been found to coprecipitate with the CD3 complex [12] and to be activated by TcR–CD3 triggering [13]. Lck is associated with the cytoplasmic tail of the CD4 surface molecule which forms a co-receptor with TcR–CD3 [14,15]. Although lck plays a major role

in CD4 signalling [16], it seems to be also required for normal TcR–CD3 signal transduction [17].

To avoid prolonged stimulation of the cell which could lead to malignant proliferation, tyrosine phosphorylation must be tightly controlled during cell homeostasis. Protein tyrosine phosphatases (PTPases) specifically dephosphorylate phosphotyrosine residues thereby negatively regulating tyrosine kinase signalling [18]. In T-lymphocytes, the major PTPase expressed on the plasma membrane is the CD45 antigen [19]. Studies of CD45-deficient variants of various human T-cell lines showed that CD45 is required to couple the T-cell receptor to downstream signalling events [20–22]. Moreover, anti-CD45 mAbs have been shown to regulate T- and B-cell activation [23,24]. T-cells also express at least another PTPase, Tc PTPase, a cytosolic enzyme of as yet unknown function [25]. To study the role of PTPases during T-cell activation, we have used a powerful PTPase inhibitor, vanadate peroxide (pervanadate), to alter the equilibrium between phosphorylation and dephosphorylation reactions. Mixing orthovanadate with H_2O_2 forms vanadyl hydroperoxide (V^{4+}OOH) also termed vanadate peroxide or pervanadate [26]. This compound has been proven to be a powerful activator of platelets [27] and myeloid cells [28,29] and to be an insulinomimetic agent [30]. Its effects have been shown to be associated with an important increase in tyrosine phosphorylation and with activation of the insulin-receptor tyrosine kinase [31,32]. We show that inhibition of PTPases by pervanadate in cells of the human leukaemic line Jurkat results in generation of early [tyrosine phosphorylation, lck and fyn activation, Ca^{2+} fluxes, *c-fos* induction, nuclear factor κ B (NF-

Abbreviations used: PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin 2; mAb, monoclonal antibody; TcR–CD3, T-cell antigen receptor complex; pNPP, *p*-nitrophenyl phosphate; PLC γ 1, phospholipase C γ 1; IP $_3$, inositol trisphosphate; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; PKC, protein kinase C.

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κ B) activation] as well as late [interleukin 2 (IL-2) production, IL-2 receptor α -chain and CD69 expression] events of T-cell activation. These results suggest that, although PTPases are necessary to deliver a positive signal for some PTK activity [33–35], they also play a crucial role in the negative regulation of T-cell activation.

MATERIALS AND METHODS

Antibodies and chemicals

Anti-CD3 and anti-CD25 mAbs were purchased from Immunotech (Marseille, France). Anti-CD69 mAb was from Beckton Dickinson (Mountain View, CA, U.S.A.). Reagents for IL-2 e.l.i.s.a. were given by Dr. Bernard Ferrua (INSERM U343). Phorbol 12-myristate 13-acetate (PMA), Ca^{2+} ionophore (A23187), phenylmethanesulphonyl fluoride (PMSF), orthovanadate, H_2O_2 , dithiothreitol (DTT), ovalbumin, BSA, Nonidet P40, catalase, luciferin and Indo-1-AM were purchased from Sigma (St. Louis, MO, U.S.A.). Enolase was from Boehringer (Meylan, France). All reagents for Northern-blot analysis were from Appligene (Pleasanton, CA, U.S.A.). The *c-fos* plasmid for transient transfection experiments was kindly provided by Dr. Jun Tsuji (Nagoya University, Nagoya, Japan). Antiserum against lck was a kind gift from Dr. Siegmund Fischer (INSERM U332, Paris, France). Anti-phosphotyrosine mAb 4G10, fyn antiserum and PLC γ 1 antiserum were from UBI (Lake Placid, NY, U.S.A.).

Cell culture

Cells of the human leukaemic T-cell line Jurkat were maintained in RPMI 1640 medium containing 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate and 5% fetal calf serum (Gibco, Cergy Pontoise, France). CD45-negative variants of Jurkat obtained by FACS (fluorescence-activated cell sorter) sorting have been described elsewhere [22].

Preparation of vanadate and pervanadate solutions

The vanadate solution was prepared in incubation buffer (30 mM Hepes, pH 7.5, 150 mM NaCl, 4 mM KCl, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , 10 mM glucose). Pervanadate was obtained by mixing vanadate with 1 mM H_2O_2 for 15 min at 22 °C. This was followed by the addition of catalase (200 $\mu\text{g}/\text{ml}$) to remove residual H_2O_2 . The concentration of pervanadate generated is denoted by the vanadate concentration added to the mixture [31]. A mixture containing only catalase and H_2O_2 was used as a control of pervanadate effects.

Immunoprecipitation and immunoblotting

Jurkat cells (2×10^6 per condition) were stimulated for the indicated times with various effectors at 37 °C. Reactions were stopped by cell solubilization in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM EDTA, 100 μM NaF, 10 mM Na_2VO_4 , 1 mM PMSF, 0.5% Nonidet P40) and incubated for 30 min at 4 °C. Lysates were centrifuged at 10000 *g* for 10 min at 4 °C and supernatants adjusted with concentrated SDS sample buffer. For immunoprecipitation, lysates were incubated with a rabbit antiserum against PLC γ 1 for 2 h at 4 °C, and Protein A-Sepharose beads were then added for 30 min. The precipitates

were washed three times in lysis buffer. Proteins were separated by SDS/PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA, U.S.A.) in a solution of Tris (20 mM), glycine (150 mM) and methanol (20%) at 55 V for 5 h at 4 °C.

The membrane was saturated with blocking buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5% BSA, 1% ovalbumin, 0.05% Tween 20, 0.02% NaN_3) for 18 h at room temperature and then probed with a mouse anti-phosphotyrosine mAb (4G10) at 1 $\mu\text{g}/\text{ml}$ in blocking buffer, for 2 h at room temperature. The filters were washed in TNA (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.02% NaN_3) and TNAT (TNA + 0.05% Tween 20). Bound antibodies were revealed after incubation with alkaline phosphatase-conjugated anti-mouse antibodies (1:5000 dilution in blocking buffer) for 2 h at room temperature followed by a colorimetric reaction as recommended by the manufacturer (Promega, Madison, WI, U.S.A.). In the case of PLC γ 1 tyrosine phosphorylation, immunoreactive proteins were detected by using ^{125}I -Protein A and the membrane was autoradiographed (3M Trimax films).

Kinase assay

After cell stimulation and lysis as described above (10^7 cells/condition), lck and fyn were precipitated with specific antibodies (1:100). Immune complexes were collected using Protein A-Sepharose. After three washes with lysis buffer and one with kinase buffer (30 mM Hepes, pH 6.8, 5 mM MnCl_2 , 5 mM MgCl_2), kinase assays were performed by adding 2 μCi of [γ - ^{32}P]ATP and 5 μg of acid-treated enolase, for 10 min at 25 °C. Samples were subjected to SDS/PAGE (10% gels), and phosphoproteins were analysed by autoradiography. Kinase activity was quantified by Čerenkov counting of enolase bands.

Phosphatase assay

Membrane and cytosolic fractions were prepared from stimulated cells. CD45 was immunoprecipitated from Jurkat lysates with 5 μg of mAb. PTPase activity was assayed against the phosphotyrosine analogue *p*-nitrophenyl phosphate (pNPP, 10 mM) at 37 °C by following the release of *p*-nitrophenol at 410 nm.

Measurement of Ca^{2+} fluxes

Cells at $10^7/\text{ml}$ were loaded with 3 μM Indo-1 for 30 min at 37 °C. They were then washed once, resuspended in fresh medium at 10^6 cells/ml and stored at room temperature in the dark until tested. The analysis was done on an ATC 3000 cell sorter (Odam Brucker, Wissembourg, France). The fluorescence intensity ratio 405 nm (Ca^{2+} -Indo-1 complex)/495 nm (free Indo-1) allows the evaluation of the changes in intracellular free Ca^{2+} concentration.

Northern-blot analysis

Total RNA was prepared by acid guanidium thiocyanate/phenol/chloroform extraction [36]. Portions (15 μg) were separated on a 1% agarose gel under denaturing conditions and transferred to Hybond N+ nylon membrane. The different probes were labelled by random priming. Hybridization was performed overnight at 65 °C and the filters were washed at 65 °C by the procedure of Church and Gilbert [37].

Transient transfection experiments

Cells were washed in RPMI 1640, and then in Tris-buffered saline (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM

Na₂HPO₄·7H₂O, 0.7 mM MgCl₂, 0.7 mM CaCl₂). Cells (5×10^7) were transfected with 10 µg of a plasmid carrying the *c-fos* gene promoter linked to a luciferase reporter gene, in the presence of DEAE-dextran (500 µg/ml) and heparin (10 µg/ml). After 30 min of incubation, they were cultured in RPMI 1640 supplemented with 5% fetal calf serum, in the presence of 100 µM chloroquin for 3–4 h. Cells were then washed and maintained for 24 h in RPMI 1640 plus 5% fetal calf serum at 37 °C. After stimulation by various effectors, cells were washed and solubilized in luciferase assay buffer (50 mM Hepes, pH 7.8, 5 mM ATP, 15 mM MgSO₄). Cellular extracts were prepared by repeated freezing and thawing. Luciferase activity was measured on 10 µg of cell extract in the presence of 1 mM luciferin on a photon-counting luminometer (Biolumat; Berthold).

Electrophoretic mobility-shift assay

Preparations of cellular cytosolic and nuclear fractions were made by the procedure of Osborn et al. [38], modified by Dignam et al. [39]. Briefly, Jurkat cells were harvested and washed in cold PBS. The cell pellets were resuspended in 30 µl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P40) per 10⁷ cells and incubated for 10 min at 4 °C. Nuclei were collected by a brief centrifugation in a Microfuge, and supernatants (cytosolic fractions) were removed and stored at –80 °C. Nuclear pellets were resuspended in 45 µl of buffer B (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF) per 10⁷ nuclei and centrifuged at 10000 *g* for 10 min at 4 °C. The supernatants (nuclear fractions) were transferred to new tubes and diluted in 67 µl of buffer C (20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF) per 10⁷ nuclei and stored at –80 °C.

Mobility-shift assays were performed as follows. The NF-κB probe was a synthetic double-stranded oligonucleotide containing the NF-κB-binding site of the IL-2 gene promoter (5'-GATCCAAGGGACTTTCATG-3') [40]. The NF-κB probe was end-labelled with [γ -³²P]ATP using polynucleotide kinase and incubated with nuclear extract samples for 20 min at 37 °C. The mixture was separated by electrophoresis on a 6% non-denaturing polyacrylamide gel in 0.5×TBE (Tris/borate 45 mM, EDTA 1 mM). The dried gels were subjected to autoradiography (Kodak X-OMAT films).

Surface staining

Cells (10⁶) were incubated in PBS containing 0.1% BSA for 30 min at 4 °C with saturating concentrations of antibodies, and then washed with the same buffer. The cells were then incubated for another 30 min period with fluorescein isothiocyanate (FITC)-conjugated antibodies. Cells were then washed and resuspended at 10⁶ cells/ml. Flow cytometry analysis was carried out on an ATC 3000 cell sorter.

Measurement of IL-2 secretion

Jurkat cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum at 10⁶ cells/ml. Various effectors were added and cells incubated for 18 h at 37 °C in a 5% CO₂ humidified atmosphere. Cells were then centrifuged at 800 *g* for 10 min. IL-2 was measured by a sequential sandwich e.l.i.s.a. as previously described [41]. Briefly, the lymphokine was first extracted from supernatants with a rabbit anti-(recombinant IL-

2) IgG immobilized on to polystyrene microtitre plates and revealed by an anti-IL-2 Fab' fragment conjugated to peroxidase.

RESULTS

Pervanadate inhibits cellular PTPase activity

We have examined the effects of pervanadate on Jurkat PTPase activities towards a phosphotyrosine analogue, pNPP (Table 1). Pervanadate abolished total as well as cytosolic and membrane PTPase activities in a dose-dependent manner. Its effect was maximal at 100 µM. Because CD45 is the most abundant PTPase on the T-cell surface, we tested the effects of pervanadate on CD45 activity. Similarly to its effect on total PTPase activities, pervanadate inhibited the activity of immunoprecipitated CD45 in a dose-dependent manner. Intact Jurkat cells had a low ecto-pNPP hydrolysis activity which was not inhibited by pervanadate (not shown). This activity may correspond to alkaline phosphatase and represented less than 20% of membrane activity as calculated in CD45-deficient cells [22]. It is unlikely that alkaline phosphatase is involved in pervanadate action, as its activity is not affected by the inhibitor.

Pervanadate induces tyrosine phosphorylation of Jurkat cellular proteins

After activation, for 10 min, of intact Jurkat cells with various effectors, the pattern of protein tyrosine phosphorylation in total cell lysates was analysed by Western blotting with anti-phosphotyrosine antibodies. Figure 1(a) shows that cell stimulation with pervanadate (1 mM) dramatically increased tyrosine phosphorylation of a large number of proteins (lane 3) compared with unstimulated cells (lane 1). At the same concentration, vanadate alone had little effect on tyrosine phosphorylation (lane 2). The most prominent substrates induced by pervanadate display molecular masses of 230, 210, 175, 150, 145, 120, 115, 105, 89, 72, 70, 59, 50, 42, 38, 36, 35, 30 and 22 kDa. The effect of pervanadate was dose-dependent: tyrosine phosphorylation was detectable at concentrations as low as 25 µM (lane 6) and increased steeply up to 1 mM (lane 3). In control conditions containing only catalase and H₂O₂, without vanadate (lane 7), tyrosine phosphorylation levels were similar to those of unstimulated cells, demonstrating that the increase in tyrosine phosphorylation requires generation of pervanadate.

A time-course experiment (Figure 1b) showed that tyrosine phosphorylation was noticeable 1 min after addition of pervanadate (lane 9) and peaked around 15 min (lane 12). No

Table 1 Inhibition of cellular PTPase activity by pervanadate

Intact cells were incubated with various concentrations of pervanadate, and PTPase activity was measured on total lysate or membrane or cytosolic fractions. For CD45 PTPase activity, CD45 molecules were isolated by immunoprecipitation with mAbs. The PTPase assay was performed on the phosphotyrosine analogue, pNPP, at 37 °C. The release of *p*-nitrophenol was followed at 410 nm. The data are representative of three independent experiments.

Conditions	Total PTPase activity	Cytosolic PTPase activity	Membrane PTPase activity	CD45 PTPase activity
Basal	1.294	0.520	0.349	1.053
Pervanadate				
100 µM	0.182	0.175	0.093	0.053
10 µM	0.400	0.265	0.175	0.269
1 µM	1.263	0.621	0.184	0.601

Figure 1 Induction of tyrosine phosphorylation of cellular proteins by pervanadate in Jurkat cells

(a) Dose-response effect of pervanadate. Jurkat cells were stimulated for 10 min at 37 °C with orthovanadate (VO_4) (1 mM), pervanadate (pV) at different concentrations or control (H_2O_2 + catalase). (b) Time course of pervanadate effect. Cells were stimulated for the indicated times (min) with pervanadate (500 μM). Proteins from cell lysates were separated by SDS/PAGE and transferred to Immobilon membranes for Western blotting with anti-phosphotyrosine antibodies. Binding of the antibody was revealed by an alkaline phosphatase-conjugated anti-mouse antibody.

decrease in tyrosine phosphorylation was observed up to 1 h of stimulation.

Figure 2 shows the effect of three different PTK inhibitors, namely genistein (100 μM), staurosporine (50 nM) and herbimycin A (10 μM), on the induction of tyrosine phosphorylation by pervanadate. Genistein and staurosporine were preincubated for 10 min while herbimycin A was added to the cells 18 h before stimulation. The inhibitors, especially herbimycin A, dramatically counteracted the effect of pervanadate. However, complete inhibition was not observed probably because of the large effect of pervanadate on tyrosine phosphorylation. In a control experiment, we verified that a protein kinase C (PKC) inhibitor, H7, had no effect on induction of tyrosine phosphorylation by pervanadate (not shown).

These results show that induction of tyrosine phosphorylation by pervanadate requires active PTK.

Pervanadate activates lck and fyn tyrosine kinases

Analysis of lck and fyn activity was performed on immunoprecipitates from pervanadate-treated intact Jurkat cells. As shown in Figure 3, a 10 min stimulation with pervanadate induced a 4.3- and a 2.6-fold increase in kinase activity of lck and fyn respectively. An increase in autophosphorylation level of the kinases was also observed, as well as several retarded bands corresponding to different phosphorylated forms of the kinases. The same results were obtained after treatment of the gels with KOH to hydrolyse phosphoserine and phosphothreonine residues, suggesting that phosphorylation mostly occurred on

Figure 2 Effects of PTK inhibitors on induction of tyrosine phosphorylation by pervanadate

Jurkat cells were preincubated for 5 min at 37 °C with genistein (100 μM) (lane 5), staurosporine (50 nM) (lane 4) and, for 18 h, with herbimycin A (10 μM) (lane 3) and then treated with pervanadate (200 μM) for 10 min at 37 °C. Tyrosine phosphorylation was revealed by a Western-blotting technique using anti-phosphotyrosine antibodies. Lane 1, control, no stimulation or preincubation; lane 2, stimulation with pervanadate with no preincubation.

Figure 3 Pervanadate induction of activation of lck and fyn PTKs

Jurkat cells were stimulated or not with pervanadate (pV; 200 μM) for 10 min at 37 °C. fyn and lck were precipitated using specific antisera. Non-immune rabbit serum was used as a control. Kinase assays were performed in the presence of [γ - ^{32}P]ATP and enolase as exogenous substrate. Samples were separated by SDS/PAGE and phosphoproteins visualized by autoradiography. Numbers correspond to increase in kinase activity as quantified by Čerenkov counting of enolase bands.

tyrosine residues (not shown). As judged by Western blotting or FACS analysis with kinase-specific antibodies, pervanadate did not modify lck and fyn levels (not shown), demonstrating that

Figure 4 Pervanadate induction of Ca^{2+} mobilization

(a) Jurkat cells were labelled with the fluorescent Ca^{2+} indicator Indo-1 ($3 \mu\text{M}$) for 30 min and stimulated with anti-CD3 mAb ($0.5 \mu\text{g/ml}$) ($\square$), $200 \mu\text{M}$ pervanadate ($\blacksquare$) or a control solution containing only catalase and H_2O_2 ($\circ$). (b) Effect of different concentrations of pervanadate. Indo-1-loaded Jurkat cells were stimulated with anti-CD3 mAb ($0.5 \mu\text{g/ml}$) ($\square$), different concentrations of pervanadate [$10 \mu\text{M}$ ($\triangle$), $100 \mu\text{M}$ ($\bullet$), 1 mM ($\blacksquare$)] or a control solution containing only catalase and H_2O_2 ($\circ$). The fluorescence intensities at 405 nm and 495 nm were analysed on a cell sorter. The ratio 405/495 is displayed which corresponds to the intracellular free Ca^{2+} concentration.

the effect we observed was due to an increase in their specific activity.

Pervanadate induces Ca^{2+} influx and stimulates tyrosine phosphorylation of $\text{PLC}\gamma 1$

We then analysed the induction of Ca^{2+} fluxes in Indo-1-loaded Jurkat cells. Figure 4(a) shows that stimulation of the T-cell receptor with anti-CD3 mAb resulted in an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), corresponding to a combination of a release from intracellular stores and an influx of external Ca^{2+} into the cell. Pervanadate also caused a rise in $[\text{Ca}^{2+}]_i$, which was slower than that observed with CD3 stimulation, but reached the same maximal value after 4–5 min. Control stimulation without vanadate failed to induce any inward flux of Ca^{2+} . The effect of pervanadate on $[\text{Ca}^{2+}]_i$ was dose-

Figure 5 Effect of EGTA on Ca^{2+} mobilization by anti-CD3 mAb and pervanadate

Jurkat cells were labelled with the fluorescent Ca^{2+} indicator Indo-1 ($3 \mu\text{M}$) for 30 min and preincubated with ($\blacksquare$) or without ($\square$) 2 mM EGTA for 5 min, and then stimulated with $0.5 \mu\text{g/ml}$ of anti-CD3 mAb (a) or pervanadate ($200 \mu\text{M}$) (b). The fluorescence intensities at 405 nm and 495 nm were analysed on a cell sorter.

dependent, with an EC_{50} of approximately $50 \mu\text{M}$ (Figure 4b). As observed for tyrosine phosphorylation, pervanadate appeared to be more efficient than vanadate in increasing $[\text{Ca}^{2+}]_i$ (results not shown). In the presence of the Ca^{2+} chelator EGTA, CD3 stimulation resulted in a transient increase in $[\text{Ca}^{2+}]_i$ corresponding solely to the release of Ca^{2+} from intracellular stores, the Ca^{2+} -influx contribution being abrogated (Figure 5a). In contrast, EGTA abolished the Ca^{2+} response induced by pervanadate (Figure 5b), demonstrating that this agent stimulated a Ca^{2+} influx only. Release of Ca^{2+} from intracellular stores is mediated by IP_3 which results from hydrolysis of phosphoinositol bisphosphate by $\text{PLC}\gamma 1$. We thus studied the effect of pervanadate on $\text{PLC}\gamma 1$ tyrosine phosphorylation by anti-phosphotyrosine Western blotting (Figure 6). Stimulation with $200 \mu\text{M}$ pervanadate for 2 min increased tyrosine phosphorylation of $\text{PLC}\gamma 1$ (Figure 6a). Under the same conditions, pervanadate had no effect on the amount of $\text{PLC}\gamma 1$ as determined by immunoprecipitation from [^{35}S]methionine-labelled cells (Figure 6b). Interestingly, a more slowly migrating

Figure 6 Pervanadate induction of tyrosine phosphorylation of PLCγ1

Jurkat cells were stimulated for 2 min with 200 μ M pervanadate (pV). After cell lysis, PLCγ1 was immunoprecipitated. Non-immune rabbit immunoglobulins were used as control. Samples were subjected to SDS/PAGE and immunoblotted with an anti-phosphotyrosine antibody (a). (b) PLCγ1 was immunoprecipitated from ³⁵S-labelled cells and analysed by SDS/PAGE.

Figure 7 Induction of tyrosine phosphorylation by pervanadate in CD45-negative Jurkat variants

Jurkat cells and CD45-negative variants were stimulated by pervanadate (pV; 200 μ M) for 10 min at 37 °C. Tyrosine phosphorylation was revealed by Western blotting with anti-phosphotyrosine antibodies.

band appeared which probably corresponds to a phosphotyrosine form of PLCγ1.

The effect of pervanadate and anti-CD3 mAb on $[Ca^{2+}]_i$ were equally blocked by preincubation with PTK inhibitors such as staurosporine (50 nM), genistein (100 μ M, 5 min) and herbimycin A (10 μ M, 18 h) (results not shown). Together, these observations show that pervanadate and anti-CD3 mAb regulate $[Ca^{2+}]_i$ by different mechanisms that both involve active PTK entities.

Figure 8 Induction of Ca^{2+} influx by pervanadate in CD45-negative Jurkat variants

Jurkat cells (open symbols) and CD45-negative variants (closed symbols) were loaded with Indo-1 and stimulated with anti-CD3 (0.5 μ g/ml) (Δ , $\blacktriangle$) or pervanadate (200 μ M) ($\square$, $\blacksquare$). Fluorescence ratios were analysed on a cell sorter.

Effect of pervanadate on a CD45-negative variant of Jurkat cells

Because the steady-state level of phosphotyrosine on cellular proteins is the result of opposing activities of PTKs and PTPases and considering that CD45 is the predominant PTPase on the surface of T-cells, we studied the effects of pervanadate in a CD45-deficient variant of Jurkat cells. First, we compared tyrosine phosphorylation levels in Jurkat cells and the CD45-negative variant (Figure 7). On unstimulated variant cells we noted the additional presence of a phosphotyrosine-containing 55 kDa protein and the absence of a 67 kDa protein. Stimulation by pervanadate led to an increase in tyrosine phosphorylation in both cell types. The tyrosine-phosphorylated protein pattern resulting from pervanadate treatment appeared to be markedly lower in the CD45-negative variant than that observed in parental cells. Interestingly, some phosphotyrosine proteins were missing in the variant (arrowheads) which could correspond to specific CD45 substrates.

These results indicate that the absence of the CD45 tyrosine phosphatase profoundly altered the effect that pervanadate exerts on tyrosine phosphorylation of several substrates.

We next analysed Ca^{2+} fluxes in the CD45-negative variant (Figure 8). As previously reported [22], these variants display a lower response to TcR-CD3 stimulation due to a deficient coupling of the receptor with downstream signalling events. The Ca^{2+} response to pervanadate was completely abolished in CD45-deficient cells even though a slight but significant response was still observed with CD3 stimulation.

Pervanadate induces *c-fos* mRNA accumulation

Transcription of the early gene *c-fos* was investigated by Northern-blot analysis of total RNA prepared from Jurkat cells (Figure 9). The level of RNA encoding the *c-fos* product largely increased after treatment of cells with PMA. Pervanadate also induced *c-fos* mRNA, but at a lower level. No synergistic effect was observed with a combination of pervanadate and PMA. The treatments did not affect actin mRNA levels. We then analysed expression of the *c-fos* gene by using a gene-reporter technique. Jurkat cells were transfected with a plasmid carrying a luciferase indicator gene under the control of the *c-fos* promoter. Activation of this promoter results in luciferase production. Luciferase

Figure 9 Northern-blot analysis

Jurkat T-cells were incubated for 30 min with 200, 100 and 50 μ M pervanadate (pV) or 50 ng/ml PMA. Total RNA was isolated, RNA blots were prepared from equalized samples (15 μ g) and separately hybridized with probes specific for *c-fos* or actin.

Table 2 Activation of the *c-fos* promoter by pervanadate and PMA

Jurkat cells were transfected by the DEAE–dextran method with a *c-fos* promoter–luciferase gene construct and grown for 72 h. The cells were then stimulated with pervanadate (200 μ M) or PMA (50 ng/ml) for 18 h. Cellular extracts were prepared by repeated freezing and thawing. Luciferase activity was measured on 10 μ g of cell extracts and was defined in relative luminescence light units. The increases in activity compared with unstimulated cells are shown in parentheses. The data are representative of three independent experiments.

Conditions	Luciferase activity (units/10 μ g of protein)
Basal	9.8
PMA	36.3 (3.7)
Pervanadate	30.98 (3.1)
Pervanadate + PMA	169.2 (17)

activity, as measured by photon counting, is proportional to luciferase concentration and therefore to *c-fos* promoter activity. The results of such an experiment are shown in Table 2. PMA

and pervanadate stimulated to the same extent (3–4-fold) the *c-fos* promoter. Maximal stimulation was obtained with a combination of pervanadate and PMA (17-fold). The compounds thus acted at a transcriptional level on the *c-fos* promoter. The synergy between PMA and pervanadate on *c-fos* promoter was not observed on *c-fos* mRNA levels. The difference could be attributed to the nature of the experiments: luciferase assays were performed after 18 h of stimulation to allow a sufficient accumulation of luciferase activity whereas cells were activated for only 30 min for Northern-blot analysis.

Induction of surface expression of the activation antigen CD69 by pervanadate

Stimulation of the T-cell receptor results in expression of a large number of genes within a long time frame [42]. One such gene encodes the early-activation antigen CD69, the surface expression of which is induced within hours of TcR–CD3 stimulation [43]. Although the function of CD69 in T-cell activation is not well understood, this antigen is a good marker of distal signal-transduction events. Flow-cytometry analysis revealed that CD69 labelling on unstimulated cells (Figure 10a) displayed a fluorescence intensity similar to that observed when an irrelevant mAb is used (not shown). Stimulation with pervanadate resulted in only a marginal increase in CD69 levels (Figure 10b). PMA was more efficient at inducing CD69 expression (Figure 10c) and its action was synergized when used in combination with pervanadate (Figure 10d). Induction of CD69 by pervanadate was blocked by herbimycin A (10 μ M) (not shown).

Pervanadate stimulates IL-2 production and surface expression of IL-2 receptor α -chain

One of the most commonly used criteria to assess late T-cell-activation events is production of the lymphokine IL-2 [44]. In Jurkat cells, induction of IL-2 gene expression requires engagement of the T-cell-antigen receptor by agonist mAb (generally anti-CD3 mAb) together with a second signal provided by the phorbol ester PMA. Stimulation of the TcR–CD3 complex can be mimicked by a Ca^{2+} ionophore A23187. As shown in Table 3, co-stimulation of Jurkat cells with Ca^{2+} ionophore and

Figure 10 Pervanadate induction of CD69 expression

Cells were stimulated or not (a) for 5 h at 37 $^{\circ}$ C by pervanadate (200 μ M) (b), PMA (10 ng/ml) (c) or a combination of the two effectors (d). Cells were first incubated with an anti-CD69 mAb, and then with an FITC-conjugated secondary antibody. Flow-cytometry analysis was performed on a cell sorter.

Table 3 Induction of IL-2 production by pervanadate

After incubation with various effectors for 18 h at 37 °C, IL-2 was measured by an e.l.i.s.a. Effectors were used at the following concentrations: PMA, 10 ng/ml; anti-CD3 mAb, 1 µg/ml; Ca²⁺ ionophore A23187, 0.2 µM. In the second experiment, pervanadate was used at 200 µM. Data are means ± S.D. of three determinations.

Experiment 1		Experiment 2	
Conditions	[IL-2] (ng/ml)	Conditions	[IL-2] (ng/ml)
Basal	0.003	Basal	0.021
A23187 + PMA	35 ± 2	A23187 + PMA	13.3 ± 0.013
PMA	0.181 ± 0.0015	Pervanadate	2.15 ± 0.35
Pervanadate (200 µM) + PMA	30 ± 2	CD3	0
Pervanadate (100 µM) + PMA	16 ± 0.02	Pervanadate + CD3	4.92 ± 0.34
Pervanadate (50 µM) + PMA	10.92 ± 2.08	Pervanadate + PMA	11.32 ± 0.3
Control + PMA	0.224 ± 0.02	Pervanadate + CD3 + PMA	16 ± 0.23
Pervanadate (200 µM)	6.98 ± 0.99	CD3 + PMA	9.21 ± 0.25
Pervanadate (100 µM)	1.01 ± 0.05		
Pervanadate (50 µM)	0.349 ± 0.033		
Control	0.002		

Table 4 Induction of surface expression of IL-2 receptor by pervanadate and PMA

Jurkat cells were stimulated for 18 h with PMA (10 ng/ml) or pervanadate (200 µM). Cells were first incubated with an anti-CD25 mAb, and then with an FITC-conjugated secondary antibody. Flow-cytometry analysis was performed on a cell sorter. These data are representative of three independent experiments.

Conditions	Percentage of cells expressing CD25 antigen
Basal	0
Pervanadate	9
PMA	22
Pervanadate + PMA	45

PMA for 18 h led to maximal secretion of IL-2 (35 ng/ml). Pervanadate alone or in combination with PMA induced a dose-dependent IL-2 production. It is notable that pervanadate, when used alone, was able to induce IL-2 secretion, although at a low level, whereas PMA alone failed to do so. In a second experiment, we compared the effect of anti-CD3 mAb and pervanadate on IL-2 production. Anti-CD3 mAb alone had no effect, whereas pervanadate alone (200 µM) was able to induce IL-2 production (2 ng/ml). When added together, the two compounds had a synergistic effect (5 ng/ml). Co-stimulation of cells with either anti-CD3 mAb and PMA or pervanadate and PMA resulted in similar levels of IL-2 secretion (respectively 9 and 11 ng/ml). Higher IL-2 production was obtained with the combination anti-CD3 + PMA + pervanadate (16 ng/ml). These results indicate that pervanadate induced IL-2 production alone or in synergy with PMA or anti-CD3 mAb.

Figure 11 Pervanadate induction of NF-κB DNA-binding activity in Jurkat T-cells

Nuclear extracts were prepared from control cells or cells stimulated for 1 h with Ca²⁺ ionophore A23187 (0.5 µM), PMA (10 ng/ml) and different concentrations of pervanadate (pV). An electrophoretic mobility-shift assay was performed with a radiolabelled oligonucleotide corresponding to the NF-κB-binding site of the IL-2 gene. The positions of the unbound oligonucleotide (free probe) and the retarded DNA-protein complex are indicated by arrows.

We then analysed another late event of T-cell activation: expression at the surface of the IL-2 receptor α-chain (CD25) [45]. The results of a flow cytometry analysis are shown in Table 4. No IL-2 receptor could be detected on unstimulated cells. Stimulation with pervanadate induced a small expression of CD25 (9% of positive cells). With PMA, levels of CD25 expression were higher (22%). Maximal induction of CD25 expression was produced by co-stimulation with pervanadate and PMA (45%). Together, these results show that inhibition of PTPases by pervanadate induced several late events of the T-cell activation process.

Pervanadate activates NF-κB

We finally investigated activation of NF-κB, one of the nuclear factors involved in the regulation of IL-2 gene transcription [46,47]. NF-κB is a heterodimer composed of two proteins, p50 and p65 [48,49]. In unstimulated cells, with the notable exception of B-cells, NF-κB is retained in the cytoplasm in an inactive complex by an inhibitory protein, IκB, and cannot bind DNA [50,51]. In response to various stimuli, the IκB-NF-κB complex dissociates, and NF-κB-binding activity is detected in the nucleus [52]. Activation of NF-κB-binding activity was measured in nuclear extracts from Jurkat cells stimulated with various effectors. As shown in Figure 11, a 1 h stimulation with A23187 plus PMA resulted in the appearance of two retarded bands corresponding to NF-κB. Stimulation with PMA alone also resulted in NF-κB-binding activity. Similarly, pervanadate could activate NF-κB in a dose-dependent manner and could potentiate

the effect of PMA. Control stimulation containing only H_2O_2 and catalase, without vanadate, failed to activate NF- κ B, demonstrating that the activation of NF- κ B requires generation of pervanadate (not shown). The effect of pervanadate on NF- κ B DNA-binding activity was insensitive to addition of EGTA to the medium at concentrations that blocked pervanadate-induced Ca^{2+} fluxes. We verified that NF- κ B DNA-binding activity was insensitive to H7, a PKC inhibitor, which prevented PMA-induced NF- κ B activity. However, the effect of pervanadate on NF- κ B was abolished by herbimycin A, a tyrosine kinase inhibitor (results not shown). These results suggest that protein tyrosine phosphorylation may have an important role in regulation of NF- κ B activation by a pathway that excludes the classical PKC-dependent regulation.

DISCUSSION

In this paper, we have studied the role of PTPases in the regulation of T-cell activation, by using a powerful inhibitor of this family of enzymes (pervanadate). Pervanadate appeared to have stimulatory effects on various cell types including platelets, peripheral T-cells, neutrophils, adipocytes and tumour cell lines such as HL60 and CHO. It is known that pervanadate acts by increasing tyrosine phosphorylation of cellular proteins [26–32, 53–55]. We first verified that pervanadate inhibited total Jurkat cellular PTPase activity as well as that of immunoprecipitated CD45.

We demonstrate here that PTPases are negative regulators of T-cell activation processes, as their inhibition by pervanadate induced early, as well as late, events of Jurkat activation. The earliest detectable effect of pervanadate stimulation was an increase in tyrosine phosphorylation of a large number of cellular substrates. Pervanadate appeared to be the most powerful promoter of tyrosine phosphorylation so far tested in human T-cells, allowing the visualization of numerous additional PTK/PTPase substrates, which were not observed with TcR–CD3 stimulation. An earlier study [56] has shown that inhibition of T-cell PTPases by phenylarsine oxide enhanced TcR-induced tyrosine phosphorylation of cellular proteins. However, distal events could not be investigated in that study owing to the toxic effect of phenylarsine oxide. The rapid and extensive effect of pervanadate on Jurkat cells demonstrates that PTPases must normally exert a tight control over tyrosine phosphorylation in T-lymphocytes. It is not yet known whether the action of pervanadate results from inhibition of PTPases that oppose constitutively active PTK or indirect stimulation of PTK through unrestrained autophosphorylation. In the first situation, the increased tyrosine phosphorylation results from the accumulation of phosphate in the absence of dephosphorylation reactions without any variation in kinase activities. In contrast, the second situation implies an increase in the specific activity of the kinases. This seems to be the case in our model, at least for the src-like PTKs lck and fyn, the activities of which are stimulated by pervanadate. This stimulation is probably involved in the increase in tyrosine phosphorylation of Jurkat cellular proteins induced by pervanadate. Our results, obtained on intact cells, thus suggest that certain PTPases exert a negative effect on lck and fyn activities. One attractive candidate is CD45, the major transmembrane PTPase expressed in T-cells. However, CD45 has been shown, at least *in vitro*, to activate lck and fyn through dephosphorylation of an inhibitory C-terminal phosphotyrosine residue (Tyr-505 and Tyr-531 for lck and fyn respectively) [33–35]. According to this model, inhibition of CD45 by pervanadate would rather inhibit lck and fyn. Never-

theless, two reports have also described an increased tyrosine kinase activity of lck in CD45-negative variants of HPB ALL [57] and Jurkat cells [58], indicating that CD45 could negatively regulate lck. Along the same line, we also measured a higher activity for lck and fyn in our CD45-negative variants (V. Imbert and J.-F. Peyron, unpublished work). Regulation of the kinases is thus likely to be more complex than the simplistic control of the phosphorylation status of Tyr-505 and Tyr-531. It is more likely that several PTPases, including CD45, with possibly opposite effects, act on the kinases through modulation of different tyrosine-phosphorylation sites. The role of individual PTPases such as CD45 in this response remains to be clarified. A direct stimulatory effect of pervanadate on tyrosine kinases can be ruled out, as we did not observe any increase in kinase activity of purified lck (not shown). In addition, pervanadate has been reported to be inactive on insulin and epidermal growth factor receptors bearing tyrosine kinase activity [27,32].

We observed that cell stimulation with pervanadate resulted in an increased intracellular Ca^{2+} concentration and we showed that it was due to entry of Ca^{2+} ions from the extracellular medium. The response was blocked by PTK inhibitors (not shown) ruling out a trivial inhibition of a Ca^{2+} ATPase by pervanadate. The Ca^{2+} response induced by pervanadate appeared to be different from that induced by anti-CD3 mAb which triggered both a mobilization of Ca^{2+} from intracellular stores and an influx. This suggests that the effect of pervanadate may be independent of IP_3 production and PLC γ 1 phosphorylation but may instead be mediated by direct tyrosine phosphorylation of a Ca^{2+} channel or of a channel-regulatory molecule. In support of this hypothesis, it has recently been demonstrated that T-cells transfected with the constitutive PTK *v-src* displayed a higher Ca^{2+} baseline and a higher Ca^{2+} response to T-cell receptor stimulation, whereas IP_3 levels were not affected [59]. However, pervanadate did stimulate tyrosine phosphorylation of PLC γ 1, even though it failed to induce Ca^{2+} release from internal stores. Our data suggest that tyrosine phosphorylation of PLC γ 1 is not sufficient by itself to activate the enzyme. PLC γ 1 activation seems to necessitate TcR–CD3 aggregation which pervanadate may be unable to induce.

Looking to more distal events, we observed that pervanadate as well as PMA were able to induce *c-fos* mRNA expression by acting, at least in part, at a transcriptional level. Pervanadate also triggered cell surface expression of two activation markers, CD69 and IL-2 receptor α -chain (CD25), and this effect was potentiated by PMA.

Secretion of IL-2 appears to be an important distal marker of cell activation. IL-2 production by Jurkat cells requires the combination of two types of stimuli [60]. Whereas one signal is produced by triggering the T-cell receptor, i.e. with anti-CD3 mAb or by the use of a Ca^{2+} ionophore, the other is provided by the phorbol ester, PMA. We report here that pervanadate was able by itself to induce IL-2 production, although at a low level. Yet, when used in combination with either anti-CD3 mAb or PMA, its effect was amplified and produced maximal levels of IL-2. This suggests that induction of tyrosine phosphorylation is sufficient *per se* to initiate a complete activation process. However, alternative pathways, for instance mimicked by PMA, are required to amplify the response.

Pervanadate was found to activate NF- κ B, one of several transcription factors that regulate transcription of the IL-2 gene [46,61]. The effect required generation of pervanadate and was not due to residual H_2O_2 , a potent inducer of NF- κ B-binding activity [62]. It was independent of Ca^{2+} and PKC and was prevented by the tyrosine kinase inhibitor herbimycin A (results not shown). Similar results have been recently reported, showing

that IL-1 may activate NF- κ B via an unknown signalling mechanism that excludes oxygen radicals and PKC activity [63]. Phosphorylation reactions have been proposed to play a role in inducing dissociation of NF- κ B from its cytosolic inhibitor I κ B [51], but to date no direct links have been suggested between tyrosine kinases and the transcription factor. The results obtained with a PTPase inhibitor suggest that tyrosine phosphorylation may play a role in NF- κ B activation. This potential role is currently under investigation in our laboratory.

In a first attempt to define more clearly the roles played by different cellular PTPases in the control of T-cell activation events, we made use of CD45-deficient variants. Interestingly, the variants failed to elicit Ca²⁺ influx in response to pervanadate. Among the various PTPases that are likely to be inhibited by pervanadate, CD45 probably plays a pivotal role in the regulation of the Ca²⁺ influx during cell activation. Inhibition of CD45 phosphatase activity may stabilize a phosphotyrosine site on an active kinase or substrate which act as positive regulators of Ca²⁺ influx. Moreover, in the CD45-negative variant, pervanadate failed to induce tyrosine phosphorylation of several cellular proteins that may represent specific CD45 substrates. Some of them may be involved in the regulation of Ca²⁺ fluxes.

Together, the data are in agreement with the potential function of PTPases as anti-oncogene products, where loss of a PTPase gene or inhibition of the product may be one of the steps leading to malignant cell transformation [64,65]. The identification of the PTPases affected by pervanadate as well as their substrates is likely to give important clues on the mechanisms regulating T-lymphocyte activation and proliferation. Pervanadate appears to be a powerful pharmacological agent for dissecting cellular activation events that are under the control of tyrosine phosphorylation.

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