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Tyrosine phosphorylation in T cells is regulated by phosphatase activity: Studies with phenylarsine oxide

(T-cell activation/protein-tyrosine-phosphatase/protein-tyrosine kinase)

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ABSTRACT **Activation of T cells induces rapid tyrosine phosphorylation on the T-cell receptor ζ chain and other substrates. These phosphorylations can be regulated by a number of protein-tyrosine kinases (ATP: protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) and protein-tyrosine-phosphatases (protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48). In this study, we demonstrate that phenylarsine oxide can inhibit tyrosine phosphatases while leaving tyrosine kinase function intact. We use this reagent to investigate the effect of tyrosine phosphatase inhibition in a murine T-cell hybridoma. Increasing concentrations of phenylarsine oxide result in an increase in tyrosine phosphate on a number of intracellular substrates in unstimulated T cells, suggesting that a protein-tyrosine kinase is constitutively active in these cells. The effect of phenylarsine oxide on T cells stimulated with an anti-Thy 1 monoclonal antibody is more complex. At low concentrations of drug, there is a synergistic increase in the level of tyrosine phosphate on certain cellular substrates. At higher concentrations, anti-Thy 1-stimulated tyrosine phosphorylation is inhibited. These results indicate that tyrosine phosphorylation in T cells is tightly regulated by tyrosine phosphatases. Partial inhibition of these enzymes results in enhanced substrate phosphorylation. Inhibition of all stimulated tyrosine phosphorylation by high doses of phenylarsine oxide suggests that tyrosine kinase activity is regulated by tyrosine phosphatases.**

Over the past decade there has been intense study devoted to understanding cellular tyrosine phosphorylation. By investigation of normal and transformed cells, it now appears that protein-tyrosine kinases (PTK; ATP:protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) and protein-tyrosine-phosphatases (protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) regulate such critical cellular functions as growth, differentiation, and signal transduction (1, 2). Our laboratory has been interested in the function and regulation of tyrosine phosphorylation in T lymphocytes. We demonstrated that activation of T cells by a variety of means results in tyrosine phosphorylation of the T-cell antigen receptor (TCR) on the TCR ζ chain (3–5). Furthermore, we have observed that other cellular substrates are phosphorylated on tyrosine more rapidly than the TCR ζ chain (5, 6). Phosphorylation of these proteins can be detected by immunoblotting with specific anti-phosphotyrosine antibodies within seconds of TCR engagement.

The PTKs responsible for phosphorylation of TCR ζ and these other substrates have not been conclusively identified. Since the TCR subunits lack the necessary amino acid sequences that define kinases it has been assumed that the TCR must be activating a nonreceptor kinase such as a member of the src PTK family. In fact, T cells have been shown to express lck, fyn, and yes PTKs (7, 8). The PTK lck has been demonstrated to be noncovalently associated with the CD4 and CD8 molecules, which are T-cell-specific glycoproteins (7, 9). Monoclonal antibody-mediated cross-linking of CD4 activates lck autophosphorylation and *in vitro* substrate phosphorylation and results in TCR ζ chain tyrosine phosphorylation *in vivo* (10). Despite this effect on TCR phosphorylation, it is not likely that TCR-mediated activation occurs solely via the lck kinase. TCR cross-linking fails to activate lck and the patterns of substrate phosphorylation mediated via TCR and CD4 engagement are distinct (11). Recently, we have demonstrated that fyn is associated with the TCR, and TCR tyrosine phosphorylation occurs *in vitro* in the presence of immune complexes containing TCR and this kinase (8). The potential for interactions between fyn, lck, and other PTKs is of great interest.

Until recently, investigators have focused almost entirely on PTKs as mediators of the tyrosine phosphorylation pathway. The identity and regulation of tyrosine phosphatases were not as extensively studied. The identification of the CD45 molecule as a tyrosine phosphatase has added impetus to the biochemical study of these enzymes (12). In T cells, manipulation of the CD45 molecule by cross-linking to a number of cell-surface molecules has interesting functional effects (13). Formation of conjugates between CD45 and the CD4 molecule results in accentuation of T-cell activation, whereas CD45 cross-linking to the TCR results in inhibition. More recently, it has been shown that T-cell clones selected to be negative for CD45 fail to be activated to produce lymphokines via the TCR (14).

The above results demonstrate that T-cell activation is associated with rapid changes in the status of cellular tyrosine phosphorylation. In view of the evidence that multiple tyrosine kinases and phosphatases could regulate these events, we sought to manipulate the tyrosine phosphorylation pathway by inhibiting tyrosine phosphatase activity. This pharmacologic approach relied on addition of phenylarsine oxide (PAO) to the T cells. In the insulin receptor system, Lane, Blackshear and collaborators (15, 16) have demonstrated that this agent inhibits tyrosine phosphatase activity and reveals tyrosine phosphorylated substrates otherwise not observed. The effect on tyrosine phosphorylation in a murine hybridoma was analyzed by immunoblotting cellular lysates and immunoprecipitated TCR with anti-phosphotyrosine antibodies.

MATERIALS AND METHODS

Cell and Antibodies. The T-cell hybridoma 2B4 and the LSTRA cell lines were maintained in culture as described (3,

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Abbreviations: PAO, phenylarsine oxide; PTK, protein-tyrosine kinase; TCR, T-cell antigen receptor.

17). The A2B4-2 monoclonal antibody binds the TCR α chain on 2B4 cells (18). Anti-lck and anti-fyn antibodies recognize specific sequences in the kinase N-terminal domain (7). They were a gift of J. B. Bolen (NCI). M1/9 is an antibody that binds CD45 (19).

Cell Treatment and Protein Analysis. PAO (Aldrich) was dissolved at 100 mM in dimethyl sulfoxide. Subsequent dilutions were made into tissue culture medium. 2B4 cells were preincubated with PAO for 10 min at 37°C and then stimulated with anti-Thy 1 antibody (G7) using a 1:100 dilution of ascites for 30 min at 37°C. The 10-min incubation time gave an optimal increase in substrate phosphorylation. Cell density was kept at 10^7 cells per ml. After stimulation, cells were washed in cold phosphate-buffered saline (PBS) containing 400 μM sodium orthovanadate and 400 μM EDTA. The cells were solubilized, and postnuclear extracts were electrophoresed and transferred onto nitrocellulose (5, 6). The tyrosine phosphorylated proteins were detected with affinity-purified anti-phosphotyrosine antibodies (5, 6).

Preparation of ^{32}P-Labeled [Val^5]Angiotensin II. LSTRA cells, because of their high level of lck tyrosine kinase activity, were used for peptide labeling. The reaction mixture contained 0.5–0.7 mg of [Val^5]angiotensin II (Sigma), 2–2.5 mg of LSTRA cell membrane, and 1.5 μM [γ-^{32}P]ATP (4100–4500 Ci/mmol; 1 Ci = 37 GBq) (ICN) in high salt buffer (20). The mixture was incubated at 37°C for 60 min and the reaction was stopped by the addition of 350 μl of 3.2% trichloroacetic acid and 400 μl of bovine serum albumin. After 30 min on ice, the trichloroacetic acid-soluble supernatant was neutralized with ammonium hydroxide and gel filtered on a Bio-Rad P-2 column (1.3 × 26 cm) equilibrated with 50 mM Hepes buffer (pH 7.4). The fractions containing the labeled peptides were identified by spotting onto phosphocellulose paper (Whatman P-81). The filters were washed three times in 0.5% phosphoric acid and once in acetone and were then assayed for radioactivity.

Phosphatase and Kinase Assays. The CD45 tyrosine phosphatase was isolated by immunoprecipitation from 10^7 2B4 cells. Immune complexes were incubated in 50 μl of buffer containing 50 mM Hepes (pH 7.4), 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml. PAO was added to this reaction mixture at the indicated concentrations and the labeled [Val^5]angiotensin II was added. The reaction was stopped by the addition of 550 μl of 5% activated charcoal suspension, which adsorbed the labeled peptides but not the released phosphate. After charcoal particles were removed by centrifugation, 200 μl of the supernatant was assayed for free phosphate.

For fyn kinase activity assays, 2B4 cells were harvested, washed in cold PBS, lysed in 150 mM NaCl/50 mM Hepes, pH 7.4/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/aprotinin (10 μg/ml)/1 mM sodium orthovanadate/5 mM EDTA. After immunoprecipitation from 5 × 10^6 cells with anti-fyn antibody, the beads were washed twice in lysis buffer without EDTA and once in 100 mM NaCl/10 mM Hepes, pH 7.4. The immunoprecipitate was preincubated for 5 min with PAO in 100 mM NaCl/10 mM Hepes, pH 7.4 (50 μl). The kinase reaction was initiated in 100 mM NaCl/10 mM $MgCl_2$/2 μM ATP/10 μCi of [γ-^{32}P]ATP in 100 μl in the presence of PAO. After 20 min at room temperature, the reaction was stopped by washing in lysis buffer, and the labeled substrates were resolved on SDS/PAGE 8%. The reaction was linear over 20 min.

RESULTS

To model the effects of PAO on the tyrosine phosphorylation pathway in T cells, we tested it on several kinases and phosphatases that are known to be present in the murine T-cell hybridoma 2B4. We first evaluated the effect of PAO on CD45, the predominant T-cell membrane tyrosine phosphatase. The 2B4 cells were solubilized in nonionic detergent and CD45 antibodies were immunoprecipitated with anti-CD45 antibody. The immunoprecipitated CD45 was then incubated with phosphotyrosine [Val^5]angiotensin II in the presence of increasing concentrations of PAO. In this assay, the release of free phosphate was measured to determine phosphatase activity. Marked inhibition of CD45 tyrosine phosphatase activity was observed in this assay with an IC_{50} between 5 and 10 μM (Fig. 1).

The drug was also added to *in vitro* protein tyrosine kinase assays, and the effect of PAO in kinase autophosphorylation was assessed. The fyn and lck PTKs were isolated with specific anti-peptide antisera. With either fyn or lck kinases (data not shown), there was no inhibition of autophosphorylation with increasing concentrations of PAO (Fig. 2). There was also no reproducible stimulatory effect of the drug on either kinase. It is likely of course that other tyrosine phosphatases and kinases can be found in these T cells. We will assume, however, for this study that the effects of PAO on the CD45 phosphatase and fyn and lck kinases are representative. We attribute the changes in substrate tyrosine phosphorylations to phosphatase inhibition.

In a recent study, we demonstrated that T-cell stimulation results in rapid tyrosine phosphorylation of a 62-kDa substrate in 2B4 cells (5). In these studies, anti-Thy 1 antibody proved most useful, since activation with it does not require additional antigen-presenting cells containing tyrosine phosphorylated proteins. As shown in Fig. 3*A* (first two lanes), two phosphoproteins appear in response to anti-Thy 1 at 62 and 21 kDa. The identity of the 21-kDa protein as TCR ζ was confirmed by immunoprecipitation with monoclonal anti-TCR antibodies (Fig. 3*D*). The majority of constitutively phosphorylated substrates seen on this gel do not change upon stimulation. The addition of PAO to these cells resulted in a complex pattern of changes in both unstimulated and stimulated cells and depended on the dose of PAO. As with

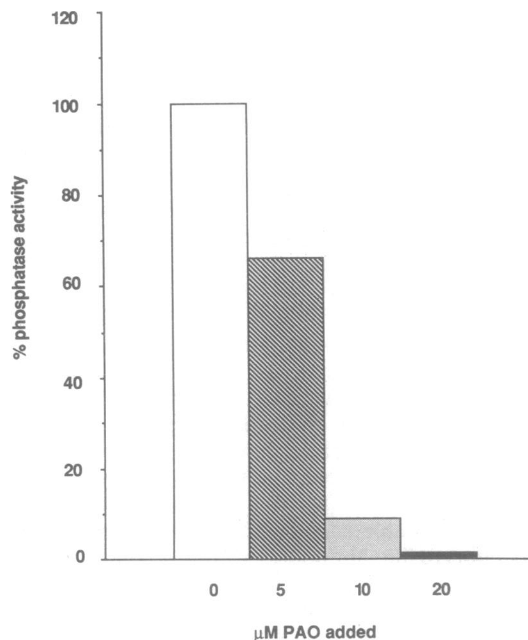

FIG. 1. CD45 tyrosine phosphatase activity is inhibited by PAO. CD45 tyrosine phosphatase activity using phosphotyrosine [Val^5]angiotensin II was tested *in vitro* in the absence or presence of different PAO concentrations. In the absence of PAO, 73 pmol of phosphate was released per min using 0.3 μM substrate. CD45 was immunoprecipitated from 10^7 2B4 cells. The indicated values represent the percentage of the activity measured in the absence of PAO.

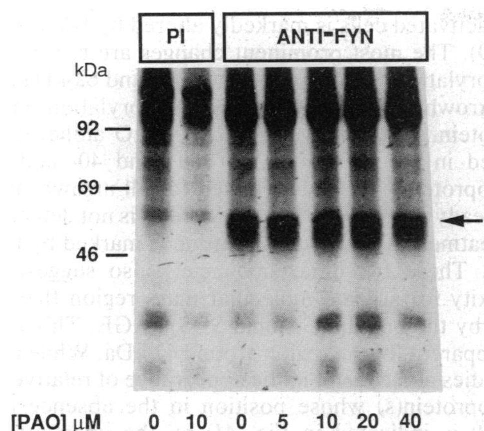

FIG. 2. Effect of PAO on PTK activity. The fyn PTK was isolated from 2B4 cells and its capacity for autophosphorylation was tested in the absence or presence of different concentrations of PAO. Kinase reactions with 5×10^6 cells per sample were carried out as described. Immunoprecipitation with preimmune serum (PI) served as a control. The position of autophosphorylated fyn is indicated by the arrow.

anti-Thy 1, most of the constitutive phosphoproteins remain unaltered at all doses of PAO. The changes produced by the addition of PAO are seen in ζ and proteins that migrate with masses of 54, 62, and 85–120 kDa (arrowheads). The effect on ζ, although apparent on the one-dimensional blot of whole cell lysates, is best appreciated in the immunoprecipitates (Fig. 3*D*). At no concentration of PAO is any ζ chain phosphorylation observed in the absence of stimulation. However, at low doses of PAO (1–3 μM), there is a severalfold increase in the level of ζ-chain phosphorylation on anti-Thy 1 stimulation. This returns to the level seen in the absence of drug at 6 μM, and ζ phosphorylation is completely inhibited above 12 μM PAO. In the absence of PAO, a very small amount of phosphoprotein at 54 kDa is seen after stimulation (Fig. 3 *A* and *B*). At 3 μM PAO, two changes in this region are seen. First, we observe phosphorylation in the absence of stimulation. This increases with increasing doses of PAO to nearly 8-fold background levels at 50 μM. Second, the combination of PAO plus anti-Thy 1 results in a massive stimulation of phosphorylation, which peaks at 12 μM PAO. The same two phenomena are observed with the 62-kDa region of the gel (Fig. 3 *A* and *C*). Finally, phosphoproteins that migrate at 85–120 kDa demonstrate dose-dependent increases in phosphorylation in response to PAO. Despite the complexity in the pattern of effects of PAO, its effects on Thy 1-mediated tyrosine phosphorylation for the three regions of the gel that demonstrate a response to Thy 1 antibodies are as follows: (*i*) at low doses of PAO, there is a synergistic increase in phosphorylation with anti-Thy 1; (*ii*) at higher doses of PAO, all anti-Thy 1-stimulated phosphorylation is

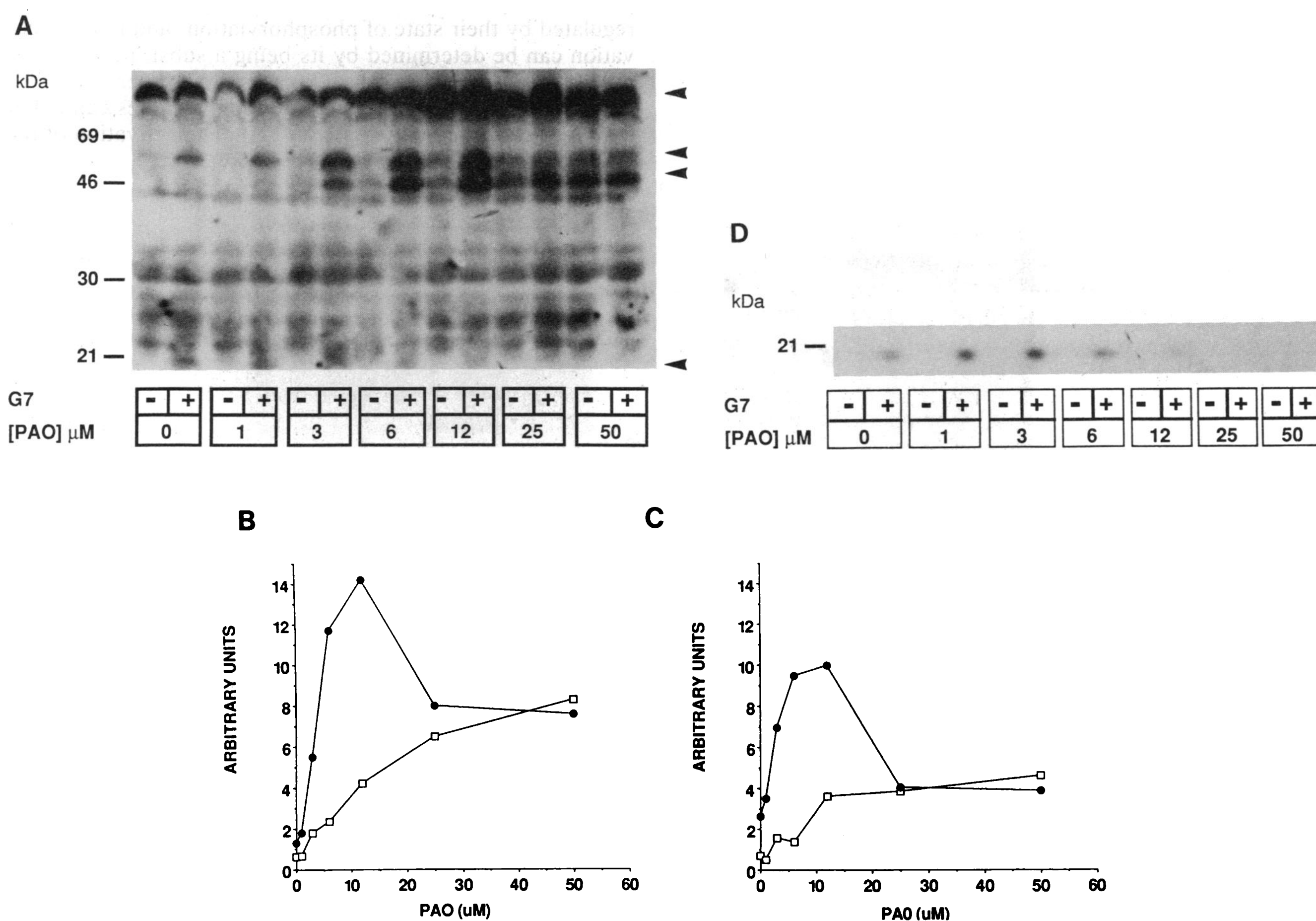

FIG. 3. Effect of PAO on substrate tyrosine phosphorylation in unstimulated and stimulated T cells. 2B4 cells were left unstimulated (lanes −) or were stimulated with a 1:50 titer of G7 ascites (lanes +). Cells were preincubated with PAO at the indicated concentrations. (*A*) Cells were stimulated, washed, and solubilized, and postnuclear extracts were subjected to electrophoresis and immunoblotting with anti-phosphotyrosine antibodies. Each lane represents an extract from 5×10^6 cells. Arrowheads indicate substrates discussed in the text. (*D*) After solubilization of 2×10^7 cells per lane, the TCR was immunoprecipitated with the A2B4-2 antibody. The eluent was electrophoresed and transferred to nitrocellulose and phosphorylated TCR ζ was detected by immunoblotting. The results of densitometric analysis of the level of tyrosine phosphorylation of pp54 (*B*) and pp62 (*C*) in unstimulated (□) and stimulated (●) cells are plotted as a function of PAO concentration.

lost. The second point is obvious for ζ but can be seen for the 54- and 62-kDa species as well (Fig. 3 *B* and *C*). Thus, although there is an enhanced level of tyrosine phosphorylation detected at even the highest doses of PAO, above 25 μM PAO, stimulation is no longer observed upon addition of anti-Thy 1. This cannot be attributed to the saturation of phosphorylation sites by PAO alone because the level of tyrosine phosphate detected at these high doses of PAO is less than that achieved with the combination of anti-Thy 1 and lower doses of PAO. As predicted from the action of PAO on vicinal sulfhydryls (15), all effects of the drug were reversed by the inclusion of 50 μM dimercaptopropanol but not 200 μM 2-mercaptoethanol (data not shown).

Two-dimensional gels have been helpful in characterizing tyrosine kinase substrates in 2B4 cells (5). The effects of PAO on unstimulated and stimulated cells were analyzed with this technique to identify substrates affected by the drug. A number of constitutively tyrosine phosphorylated substrates can be detected in unstimulated 2B4 cells and resolved on two-dimensional gels (Fig. 4*A*). Upon activation with G7 (Fig. 4*B*), we detect tyrosine phosphorylation of the TCR chain, a 62-kDa substrate, which appears as a set of spots (arrows), and an increase in the intensity of several, unresolved large (110–120 kDa) proteins (5). Two-dimensional gels reveal the complexity of the response to phosphatase inhibition in both unstimulated and G7-stimulated cells. In unstimulated cells (Fig. 4*C*), these gels reveal that PAO alone results in tyrosine phosphorylation of a 54-kDa protein with a complex focusing pattern (large arrowhead) as well as phosphorylation of an 85-kDa protein (small arrowhead) and an increase in tyrosine phosphate on the 110- to 120-kDa proteins. Several constitutively phosphorylated substrates appear not to be affected. The pattern of phosphorylation of the G7-activated cells is markedly altered by PAO treatment (Fig. 4*D*). The most prominent changes are the increase in phosphorylation of a complex set of 54- and 62-kDa proteins (large arrowheads). In addition, phosphorylation on the 85-kDa protein, revealed in Fig. 4*C* by PAO alone, is greatly enhanced in the G7-stimulated cells and 40- and 25-kDa phosphoproteins are now detected (small arrowheads). We have already noted that phosphorylated ζ is not detected after PAO treatment. Its expected position is marked by the open triangle. These two-dimensional gels also suggest greater complexity for a given molecular mass region than can be defined by the one-dimensional SDS/PAGE. This is particularly apparent for the region around 62 kDa. While anti-Thy 1 antibodies alone result in the appearance of relatively basic phosphoprotein(s) whose position in the absence of PAO (Fig. 4*B*) is indicated in Fig. 4*D* by the open arrow, the combination of this stimulus plus PAO leads to both more intense and more acidic spots (Fig. 4). At this point, we cannot distinguish between different substrates and different numbers of phosphates added to a single substrate.

DISCUSSION

It is not surprising that the phosphorylation status of proteins within a cell represents a balance between phosphorylation and dephosphorylation. However, a variety of observations suggest that the interactions between kinases and phosphatases may be more direct. The activity of kinases may be regulated by their state of phosphorylation, and kinase activation can be determined by its being a substrate for phosphatases. For several tyrosine kinases, it has been suggested that phosphorylation of specific tyrosine residues can either inhibit or enhance kinase activity (21). The activation of the

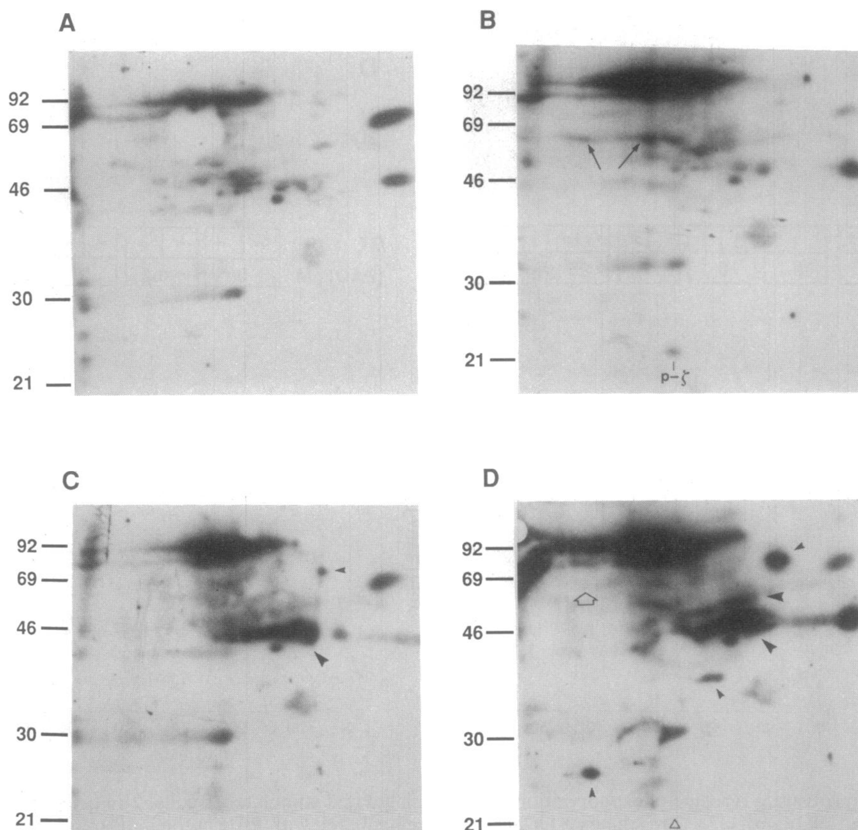

FIG. 4. 2B4 cells were left unstimulated (*A*), stimulated with anti-Thy 1 ascites (1:50) (*B*), treated with 10 μM PAO (*C*), or stimulated with G7 (1:50) and 10 μM PAO (*D*). The cells were solubilized as described and postnuclear supernatants were electrophoresed in two-dimensional isoelectric focusing SDS/polyacrylamide gel. Tyrosine-phosphorylated proteins were detected by immunoblotting. The significance of the arrows is discussed in the text.

TCR provides a fertile area for investigation into these issues. Stimulation of the TCR results in the activation of one or more nonreceptor tyrosine kinases. Recent work from our laboratory suggests that a likely candidate for the TCR-activated PTK is fyn, which is noncovalently associated with the TCR (8). The discovery that the cytoplasmic tail of CD45 is a tyrosine phosphatase demands that the role of such phosphatases in T-cell activation be addressed (12). Cross-linking of CD45 has been shown to alter signal generation in T cells. These studies have suggested that the proximity of CD45 to other surface molecules is critical to the ability of the CD45 to effect T-cell activation (13). Moreover, it appears that one T-cell-specific tyrosine kinase, lck, can be altered via CD45-mediated dephosphorylation of Tyr-505, a residue whose state of phosphorylation may correlate with kinase activity (22, 23). Finally, the characterization of T cells that lack surface CD45 has pointed to a requirement for this molecule in T-cell signal transduction (14). In this study, we have used a pharmacologic inhibitor of tyrosine phosphatases, the trivalent arsenical, PAO, to assess its impact on activation-induced tyrosine phosphorylation of a variety of intracellular substrates including the TCR ζ chain.

In adipocytes, PAO inhibits insulin induction of both glucose uptake and phosphorylation of several proteins on serine residues (24, 25). Lane and collaborators (15) also observed tyrosine phosphorylation of a 15-kDa protein only in the presence of both insulin and PAO. They proposed that PAO inhibited tyrosine phosphatase activity, thus revealing the 15-kDa protein as a substrate. Similarly, in fibroblasts, pretreatment with PAO reveals multiple additional insulin-induced tyrosine phosphorylations not seen with insulin alone (16). Such data support the concept proposed by Lane and colleagues that there are a number of tyrosine kinase substrates whose phosphate levels are predominantly regulated by phosphatases.

As with any drug, we must be cautious in our interpretations, as we cannot identify all of its effects in intact cells. It is clear that PAO does not acutely poison the cell as it enhances levels of tyrosine phosphorylation. However, prolonged incubation with the drug resulted in cell death, precluding an analysis of the effect of PAO on lymphokine generation. We examined three critical potential targets of PAO in T cells. These were the two tyrosine kinases that have been implicated in T-cell activation, lck and fyn, and the major membrane-bound tyrosine phosphatase, CD45. While CD45 phosphatase activity is completely inhibited by PAO, neither lck nor fyn is affected by the drug. The effects of PAO on tyrosine phosphate levels for a variety of intracellular proteins are complex but can be explained in a straightforward manner. We can divide the kinases responsible for the many phosphorylations seen into two groups: receptor activated and receptor independent. In making this distinction, we are not assuming how many kinases can be in either group or, indeed, whether the same kinases are in both groups. Rather it is an operational definition based on the ability to observe increased levels of tyrosine phosphate on substrates upon addition of the stimulatory antibodies. For receptor-activated phosphorylation events, the effects of PAO are dose dependent. At low doses, the inhibitor is synergistic with the antibody and increased levels of tyrosine phosphate are seen. As the dose of PAO is increased, the receptor-activated tyrosine phosphorylation is inhibited so that at >25 μM PAO, no receptor-stimulated tyrosine phosphorylation is observed. This concentration of drug completely inhibits CD45 activity *in vitro*. PAO also affects the level of tyrosine phosphate on a variety of substrates independent of the addition of the stimulatory anti-Thy 1 antibody. The mechanism(s) responsible for this can include the failure to remove phosphate from substrates that are the targets of constitutively active kinases or the activation of kinases by the addition of PAO either as a direct effect or via the inhibition of tyrosine phosphatases.

The ability of PAO to synergize with antibody in terms of the level of tyrosine phosphorylation most likely reflects the fact that the level of tyrosine phosphate achieved is a balance between the addition and removal of tyrosine phosphate. We have estimated that only $\approx 10\%$ of the total TCR ζ chain is tyrosine phosphorylated upon activation. However, the effect of PAO suggests that this percentage may be severalfold higher. The extraordinary differences in the level of tyrosine phosphate achieved upon activation with the addition of PAO suggests that altering tyrosine phosphatase activity can have dramatic effects on the consequences of TCR-mediated kinase activation. Finally, the fact that PAO can completely inhibit the ability to stimulate tyrosine phosphorylation at doses that do not directly inhibit fyn and lck support the model that a tyrosine phosphatase, perhaps CD45, is essential for the activation of tyrosine kinases in T cells.

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