Vanadate and Molybdate Increase Tyrosine Phosphorylation in a 50-Kilodalton Protein and Stimulate Secretion in Electropermeabilized Platelets†

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ABSTRACT: Addition of vanadate and molybdate to electropermeabilized human platelets caused a time- and dose-dependent increase in the phosphotyrosyl content of 50- and 38-kDa proteins. This effect can most likely be attributed to an inhibition of protein-tyrosine-phosphatase activity because vanadate and molybdate inhibited this activity in platelet extracts by greater than 97% while causing an increase in tyrosyl phosphorylation of artificial substrates that had been added to the same extracts. The addition of vanadate and molybdate to the electropermeabilized platelets also induced an increase in serotonin and PDGF secretion. Interestingly, the secretion of these components tightly correlated in a time- and dose-dependent fashion with the phosphorylation of the 50-kDa protein on tyrosyl residues. This suggests that the tyrosine phosphorylation of this protein may be closely linked to the platelet activation cascade.

Platelets have frequently been used as models to elucidate mechanisms of signal transduction. Most studies have focused on pathways that lead to the generation of diacylglycerol and inositol trisphosphate (IP_3),[1] which act as second messengers to promote platelet responses, or cAMP, which acts as a second messenger to inhibit them. Although each of these second messengers has been shown to promote the phosphorylation of platelet proteins on serine and threonine residues (Nishizuka, 1984), the mechanism by which each affects platelet functions is poorly understood.

The observation that high levels of protein-tyrosine kinase activity are present in platelets (Phan-Dinh-Tuy et al., 1983; Nakamura et al., 1985; Golden et al., 1986) has raised the possibility that tyrosine phosphorylation reactions also may be involved in the physiological response. Moreover, investigators in three different laboratories have recently shown that thrombin, the most potent known activator of platelets, can stimulate the phosphorylation of several different platelet proteins on tyrosyl residues (Ferrell & Martin, 1988; Golden & Brugge, 1989; Nakamura & Yamamura, 1989). It is, however, too early to assess how tyrosine phosphorylation may influence steps in the cascade of events linked to platelet activation. Protein tyrosine phosphorylation may influence responses by stimulating the degradation of phosphoinositides or by acting at a later point in the cascade to promote specific responses such as aggregation or the secretion of platelet-derived growth factor or serotonin. Clearly, many different experimental approaches will be required to sort out these and other possibilities.

The approach we used in this study takes advantage of the fact that vanadate and molybdate are potent inhibitors of tyrosine phosphatase reactions (Swarup et al., 1982). These inhibitors were added to permeabilized platelets, and their effects on the control of tyrosine phosphorylation reactions and secretory responses were examined. This approach led to the identification of a specific platelet protein of approximately 50 kDa that showed a pronounced sensitivity to vanadate and molybdate. The phosphorylation of this protein on tyrosyl residues increased in the presence of the two inhibitors and correlated closely with an induced secretion of serotonin and platelet-derived growth factor.

Experimental Procedures

Materials. Essentially fatty acid free bovine albumin, crystallized human albumin, GTPγS,[1] prostaglandin E_1, vanadium-free ATP, apyrases (grade 1), glutamic acid (K^+ salt), phosphotyrosine, phosphothreonine, and phosphoserine were from Sigma, and acrylamide, *N,N'*-methylenebis(acrylamide), and the Immuno-blot assay kit were from Bio-Rad. Prestained high molecular weight protein standards were purchased from Bethesda Research Laboratories. New England Nuclear supplied [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]myoinositol (17.1 Ci/mmol), ^{125}I-labeled protein A, and nitrocellulose. 5-Hydroxy[2-^{14}C]tryptamine creatine sulfate (54 mCi/mmol) was from Amersham, affinity-purified rabbit anti-goat immunoglobulin from Calbiochem, sodium orthovanadate from Alpha Products (Danvers, MA), and ammonium molybdate from Mallinckrodt. Goat anti-phosphotyrosine antiserum, which was produced against phosphotyramine–KLH conjugates, was a generous gift from Drs. James N. Livingston and Robert A. Mooney, University of Rochester, Rochester, NY. Protein-tyrosine-phosphatase (PTPase) was purified from human placenta as previously described (Tonks et al., 1988b).

Isolation and Permeabilization of Platelets. Human blood (10 parts) was drawn into acid/citrate/dextrose (1 part). Platelet-rich plasma was obtained following centrifugation at

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; IP, inositol 1-phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PDGF, platelet-derived growth factor; PTPase, protein-tyrosine-phosphatase; RCM lysozyme, reduced, carboxamidated, and maleylated (RCM) lysozyme; RR-src peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Gly; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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110g for 10 min at 21 °C. Prostaglandin E_1 and apyrases were added to the PRP to final concentrations of 0.4 μM and 12.5 milliunit/mL, respectively. The platelets were then removed by centrifugation at 1100g for 10 min, washed once in a modified Tyrode's buffer containing 5 mM Hepes, pH 6.4, 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 12 mM $NaHCO_3$, 0.25% (w/v) human serum albumin, 6 milliunits/mL apyrases, and 0.2 μM prostaglandin E_1, and then suspended at a concentration of 1.5×10^9 cells/mL in a similar modified Tyrode's buffer that contained 0.5 mM EGTA instead of prostaglandin E_1.

Platelet suspensions were electropermeabilized with 10 discharges of an electric field of 20 kV/cm in an apparatus identical with that used by Haslam and Davidson (1984). The suspensions were immediately placed on ice and then centrifuged at 1100g for 10 min at 4 °C. The permeabilized platelets were suspended at a concentration of 1.5×10^9/mL in ice-cold buffer containing 10 mM Hepes, pH 7.2, 140 mM potassium glutamate, 1 mM EGTA, and 0.624 mM $CaCl_2$. Under these conditions, free Ca^{2+} concentrations were calculated to be 0.3 μM using an apparent stability constant of 3.314×10^6 M^{-1} for the Ca–EGTA complex which was calculated from previously published absolute stability constants (Fabiato, 1981). These Ca^{2+} levels did not stimulate platelet release reactions in the absence of agonists. The platelet suspensions were kept on ice and used for up to 3 h after permeabilization. Electropermeabilization did not cause any detectable leakage of lactate dehydrogenase from the platelets, whereas the amount of [^{14}C]serotonin found in the suspension medium rose from approximately 35 fmol/10^9 platelets (5% of total) to 70 fmol/10^9 platelets.

Measurement of Protein Phosphotyrosine in Permeabilized Platelets. Permeabilized [^{14}C]serotonin-labeled platelets were incubated in a final volume of 62.5 μL and treated as described for the serotonin release assay except that (a) 3.3 mM [γ-^{32}P]ATP (100 cpm/pmol, 10 μCi/tube) was used instead of nonradioactive ATP, and (b) the reaction was stopped by adding 30 μL of SDS sample buffer containing 120 mM Hepes, pH 6.8, 3% SDS, 30% glycerol, 0.15% bromophenol blue, and 15% 2-mercaptoethanol. The samples were immediately boiled for 3 min and then subjected to electrophoresis in 5–15% gradient polyacrylamide gels as described by Laemmli (1970). High molecular weight standards were routinely included. The gels were fixed in 10% trichloroacetic acid, stained with 0.25% Coomassie blue R-250, destained, dried, and exposed to Kodak RP film with Dupont Cronex intensifying screens. In-gel alkali digestion of ^{32}P-phosphoproteins was performed as described by Cooper and Hunter (1981). Acid hydrolysis of ^{32}P-phosphoproteins was conducted as previously described (Tonks et al., 1988b).

Western Analysis of Platelet Proteins with Phosphotyrosine-Specific Antibodies. Permeabilized platelets were incubated as described above except that 3.3 mM unlabeled ATP was used. Similar incubations were performed with intact cells, but in the absence of external ATP, since this may interact with adenosine or ADP receptors and affect the response of intact platelets. The reactions were stopped either by adding SDS sample buffer as described above or by rapidly removing the platelets by centrifugation at 10000g and resuspending them in isoelectric focusing sample buffer containing 9 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and Ampholines. The platelet proteins were first separated by one- or two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) and then immediately transferred electrophoretically onto nitrocellulose. The nitrocellulose was incubated 1 h at 21 °C successively with (a) 3% gelatin in 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl (TBS), (b) primary antibody solution which contained 2% bovine serum albumin and goat anti-phosphotyrosine antiserum (1:1000 dilution), and (c) rabbit anti-goat IgG antibody (4 μg/mL). The primary antibody incubations were carried out in the absence or presence of 100 μM phosphoserine, phosphothreonine, or phosphotyrosine. The bound anti-phosphotyrosine antibody was then detected by using either goat anti-rabbit horseradish peroxidase conjugate (HRP) (Bio-Rad) or ^{125}I-labeled protein A. Nonspecific association of the antibody with platelet proteins, defined as the association of the antibody with proteins in the presence of an excess of phosphotyrosine, was routinely assessed.

To evaluate the amount of ^{125}I-labeled protein A that specifically associated with the antibody, autoradiograms were superimposed over the nitrocellulose, and the bands corresponding to the 50-kDa protein were excised. To assess nonspecific association of protein A, Western analysis was conducted in the presence of an excess of phosphotyrosine; the amount of radioactivity associated with the 50-kDa protein was determined and subtracted.

Phosphatase Treatment of Protein Blots. Prior to the analysis, nitrocellulose filters containing the proteins of interests were incubated in 3% gelatin and then treated for 1 h at 25 °C with either PTPase (ca. 1 unit/mL) in an imidazole buffer, pH 7.0, containing 1 mg/mL bovine serum albumin, 5 mM dithiothreotol, and 10 mM EDTA or with phosphatase 2A (ca. 2 units/mL) in an imidazole buffer, pH 7.0, containing 2 mM $MnCl_2$, 5 mM dithiothreotol, 1 mg/mL bovine serum albumin. The reactions were stopped by successively washing the membrane filters 3 times with TBS. The nitrocellulose membranes were either exposed to film or used in Western analysis as described above. A major feature of this assay, which merits comment, is that PTPase purified from placenta specifically dephosphorylated phosphotyrosyl-containing proteins immobilized on nitrocellulose. We found that the enzyme dephosphorylated ^{32}P-labeled reduced, carboxamidated, and maleylated (RCM) lysozyme and not the phosphoserine-containing substrate phosphorylase *a* (data not shown). In contrast, phosphorylase *a* was dephosphorylated by the serine/threonine phosphatase 2A. Phosphatase 2A also attacked ^{32}P-labeled RCM lysozyme which is consistent with observations that this enzyme has low PTPase activity (Goris et al., 1988).

Measurement of Protein-Tyrosine Kinase and -Phosphatase Activities in Platelet Extracts. Intact platelets were isolated as described, but suspended at a concentration of 1×10^{10} cells/mL in a buffer containing 10 mM Hepes, pH 7.2, and 140 mM NaCl. The suspension was diluted 1:5 with 10 mM Hepes, pH 7.2, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 0.025% dithiothreitol, and 100 μg/mL leupeptin (quench buffer) and then sonicated in an ice bath with two 15-s bursts at a setting of −50 with a Braun sonicator. Particulate material was removed by centrifugation at 10000g for 5 min at 4 °C and solubilized in 0.1 mL of quench buffer that contained 0.5% Triton X-100. The supernatant and particulate fractions were then assayed for PTPase by measuring the release of ^{32}P from radiolabeled RCM lysozyme as previously described (Tonks et al., 1988b) and kinase activity by measuring the tyrosine phosphorylation of RR-src peptide,[1] poly(Glu-Tyr), or RCM lysozyme.

Measurement of [^{3}H]Inositol Phosphate Formation. Human platelets do not incorporate exogenous [^{3}H]inositol into phospholipids to any great amount (Mauco et al., 1987; Culty

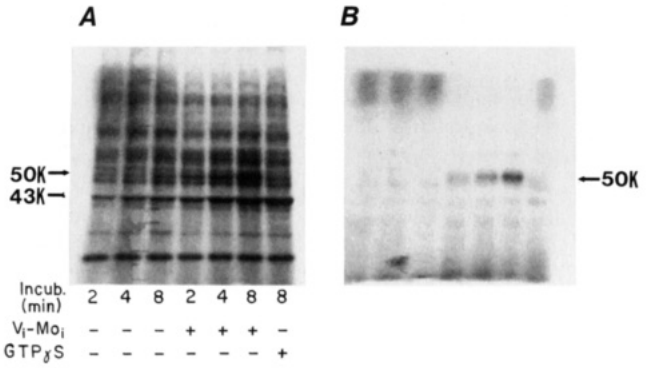

FIGURE 1: Vanadate- and molybdate-induced changes in the phosphorylation pattern of proteins in electropermeabilized platelets. (A) Platelets were permeabilized, preincubated at 25 °C for 2 min with $MgCl_2$ and [γ-^{32}P]ATP as described under Experimental Procedures, and then incubated for 2, 4, or 8 min at 25 °C with buffer, with a mixture of 100 μM vanadate plus 10 μM molybdate (V_i–Mo_i), or with 100 μM GTPγS. The reactions were stopped by the addition of SDS gel buffer, and the phosphoproteins were separated on a 5–15% polyacrylamide gel. This autoradiogram is representative of four different experiments. (B) The gel in (A) was subjected to alkali treatment as described by Cooper and Hunter (1981) and then reexposed to Kodak RP film. Similar results were obtained in two separate experiments.

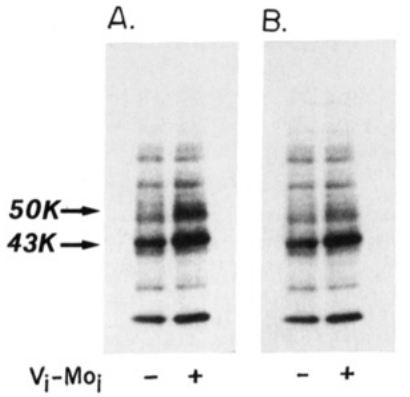

FIGURE 2: PTPase treatment of ^{32}P-labeled platelet proteins immobilized on nitrocellulose. (A) Electropermeabilized platelets were incubated with [γ-^{32}P]ATP in the presence or absence of vanadate and molybdate for 8 min as described for Figure 1. The phosphoproteins were transferred to nitrocellulose and exposed to film overnight. (B) The nitrocellulose membrane from (A) was treated with purified preparations of PTPase and reexposed to film overnight.

et al., 1988). To increase the efficiency of labeling, permeabilized platelets were incubated with [^{3}H]inositol for 2 h at 25 °C in the presence of 3.3 mM MgATP, 1 mM $MnCl_2$, and 2 mM CTP, and 1 mM EGTA. Under these conditions, the extent of labeling of the inositol phosphates increased by a factor of 10 compared to that found in intact platelets. Assay mixtures (0.5 mL) containing 1×10^9 platelets/mL were then treated with buffer alone (control), a mixture of 100 μM vanadate and 10 μM molybdate, or with 100 μM GTPγS for 8 min at 25 °C. The reactions were terminated by adding trichloroacetic acid, and the inositol phosphates were separated by Dowex chromatography as previously described (Culty et al., 1988).

Measurement of Serotonin and PDGF Release. Intact platelets in platelet-rich plasma were incubated at 37 °C in the presence of 2 mM [^{14}C]serotonin (54 mCi/mmol) for 45 min, then isolated, permeabilized, and suspended in Ca^{2+}–EGTA buffer as described above. Assays for serotonin release were conducted at 25 °C in a final volume of 62.5 μL. Platelets (6.5×10^7) were preincubated for 2 min in the presence of 5.3 mM $MgCl_2$ and 3.3 mM ATP, after which the reactions were started by adding an activating agent. At various times, the reactions were stopped by adding 0.1 volume of 37% formaldehyde, the formaldehyde-treated platelets were centrifuged at 10000g for 1 min, and the radioactivity in the supernatant was measured. The amount of [^{14}C]serotonin released is expressed as a percent of the total amount of [^{14}C]serotonin in 6.5×10^7 platelets. For PDGF measurements, reactions were conducted with unlabeled permeabilized platelets and terminated by rapid centrifugation. The levels of PDGF that were released were assessed by a radioreceptor assay (Bowen-Pope & Ross, 1985).

Results

Effect of Vanadate and Molybdate on Phosphotyrosine Content of Platelet Proteins. When vanadate and molybdate were added to electropermeabilized platelets in the presence of [γ-^{32}P]ATP, reproducible time-dependent increases in the incorporation of ^{32}P into several proteins were observed (Figure 1). As seen in this figure, proteins with apparent molecular weights of ca. 50K and 43K appeared to be phosphorylated to the greatest extent. No such effect was observed when intact platelets were used.

To determine whether vanadate and molybdate caused an increase in the content of phosphotyrosine in the 50- and 43-kDa proteins, the polyacrylamide gels from the above experiments were treated with alkali (Cooper & Hunter, 1981). Application of this technique revealed increased levels of alkali-stable phosphate only in the 50-kDa protein (Figure 1b). Consistent with this observation, phosphotyrosine (together with phosphoserine and phosphothreonine) was detected only in an acid hydrolysate of the 50-kDa protein, which was excised from polyacrylamide gels, whereas only phosphoserine and a small amount of phosphothreonine were detected in a hydrolysate of the 43-kDa protein (data not shown).

A limitation of the alkali treatment procedure is that selective loss of proteins (Cantor et al., 1987; Bourassa et al., 1988) and incomplete hydrolysis of phosphoseryl and phosphothreonyl residues can occur. Therefore, two additional approaches were used to examine the phosphotyrosine content of the platelet proteins. First, labeled phosphoproteins from polyacrylamide gels were transferred to nitrocellulose membranes and then treated with purified PTPase; only the 50-kDa phosphoprotein was dephosphorylated (Figure 2). Treatment of the nitrocellulose membranes with phosphatase 2A resulted in a dephosphorylation of all phosphoproteins (data not shown). Second, a phosphotyrosine-specific antibody was used to analyze gels by the Western procedure (Towbin et al., 1979). In these experiments, antibody binding was detected either by colorimetric means (Figures 3 and 5) or with ^{125}I-labeled protein A (Figure 4). When the approach described under Experimental Procedures was used, considerable nonspecific association of the antiserum with proteins was observed (see Figures 3–5). A parallel Western analysis was therefore conducted in each instance to assess the contribution of this nonspecific interaction by preincubating the antiserum with an excess of phosphotyrosine (+PY). As expected, the antibody specifically recognized proteins in the 50-kDa range in permeabilized platelets treated with vanadate and molybdate. The antibody did not recognize specifically proteins in permeabilized platelet preparations treated with buffer only or with GTPγS, an artificial activator of platelet functions (Figure 3). The interaction between the antibody and these proteins was completely prevented by an excess of free phosphotyrosine (Figure 3, +PY), but not by free phosphoserine and phosphothreonine (Figure 3, +PS/PT).

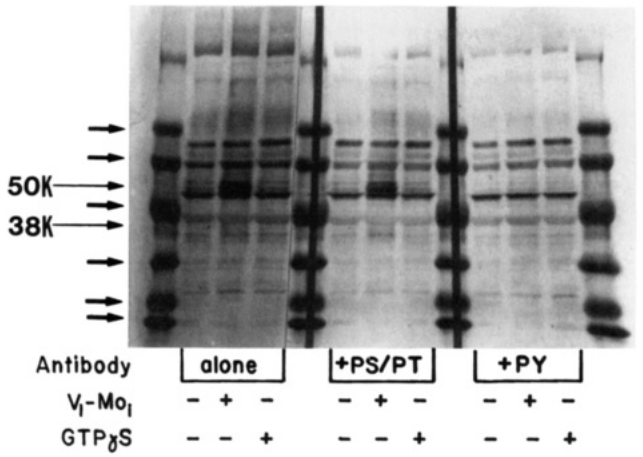

FIGURE 3: Western analysis of platelet proteins with a phosphotyrosine-specific antibody. Electropermeabilized platelets were incubated at 25 °C with buffer, a mixture of vanadate and molybdate (+V_i–Mo_i), or GTPγS for 8 and 2 min, respectively. The reactions were solubilized in SDS gel buffer and subjected to electrophoresis in a 5–15% polyacrylamide gel. After electrophoresis, the proteins were immediately transferred onto nitrocellulose and incubated with an anti-phosphotyrosine antibody alone, or in the presence of an excess of either a phosphoserine and phosphothreonine mixture (+PS/PT) or phosphotyrosine (+PY). The antibody was then detected by using the HRP conjugate method. The molecular weight markers that are indicated by arrows on the left are phosphorylase B (97K), bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen (25.7K), β-lactoglobulin (18.4K), and lysozyme (14.3K). This Western analysis is representative of three separate experiments.

When platelet proteins were separated by using a two-dimensional gel system, the antibody appeared to recognize material present in only one major spot of 50 kDa with an isoelectric point of approximately 5.2 (Figure 4). Further experiments demonstrated that the incorporation of phosphate on tyrosyl residues in proteins in the 50-kDa range was time (Figure 4) and dose dependent (Figure 5). In addition, as seen in Figure 5, the antibody recognized a 38-kDa protein in the presence of the highest concentrations of the inhibitors, i.e., 250 μM vanadate and 25 μM molybdate, and incorporation of phosphate on tyrosyl residues in this protein was also time dependent (data not shown). In agreement with those studies that employed [γ-^{32}P]ATP, no detectable change in the level of phosphotyrosine was found in intact platelets that were treated with the inhibitors.

Overall, these studies show that the addition of vanadate and molybdate to permeabilized platelets leads to an increase in phosphotyrosine content in a limited number of platelet proteins, most prominently and reproducibly in a platelet protein of 50 kDa. This contrasts with the results that others carried out with thrombin-treated platelets (Ferrell & Martin, 1988; Golden & Brugge, 1989; Nakamura & Yamamura, 1989), in which different proteins of larger molecular weight were identified.

Effect of Vanadate and Molybdate on Platelet Extracts. Vanadate and molybdate have been shown to be potent inhibitors of PTPases (Tonks et al., 1988a). To examine this property in relation to platelet PTPases, we measured the PTPase activity of platelet extracts in the presence or absence of vanadate and molybdate (Table IA). We found that platelets contain relatively high levels of PTPase compared with the levels reported for other tissues (Shriner & Brautigan, 1984; Tonks et al., 1988a). For example, the specific PTPase activity in platelet extracts, measured by using ^{32}P-RCM lysozyme as a substrate, was 2–7-fold greater than that found for the placenta or kidney extracts, which contain the highest levels of the enzyme observed to date. When sodium vanadate

Table I: Protein-Tyrosine-Phosphatase and Kinase Activities in Platelet Extracts[a]

	Part A protein-tyrosine-phosphatase act. (nmol of ^{32}P released/10^9 platelets)			
	soluble fraction		particulate fraction	
substrate	(−)	(+)	(−)	(+)
^{32}P-RCM lysozyme	8 ± 1	0.25	1.7 ± 0.3	0.03

	Part B app protein-tyrosine kinase act. in soluble extracts (nmol of ^{32}P incorporated/10^9 platelets)	
assay time (min)	(−)	(+)
5	1.2	3
10	2.0	6.2
15	2.3	9.7

[a] Platelet extracts were prepared, and the protein-tyrosine-phosphatase and kinase activities were assayed, in the presence (+) or absence (−) of 100 μM vanadate and 10 μM molybdate. (A) Phosphatase activities were measured with ^{32}P-RCM lysozyme as a substrate. These values are expressed as mean ± SE of six different experiments. (B) Kinase activities were measured with RR-src peptide (Casnellie et al., 1985) as the substrate. These results are representative of three experiments.

Table II: Effect of Vanadate and Molybdate on the Generation of Inositol Phosphates in Electropermeabilized Platelets[a]

treatment	incubation time (min)	cmp [^{3}H]IP	[^{3}H]IP_2	[^{3}H]IP_3
GTPγS	2	750 ± 200	500 ± 5	0
	4	1900 ± 50	1000 ± 50	135 ± 80
	8	4700 ± 1000	2200 ± 500	280 ± 30
vanadate + molybdate	2	400 ± 5	400 ± 50	70 ± 15
	4	750 ± 100	500 ± 100	160 ± 100
	8	1250 ± 250	1150 ± 400	200 ± 50

[a] Permeabilized platelets were labeled with [^{3}H]inositol as described under Experimental Procedures. The platelets were then incubated in the Ca^{2+}-clamped buffer for the indicated times with buffer as a control, a mixture of 100 μM vanadate and 10 μM molybdate or 100 μM GTPγS. Finally, the [^{3}H]inositol phosphates generated were separated by ion-exchange chromatography, and the radioactivity was measured. The results are expressed as the mean ± SE of two separate experiments. Each value represents cpm over control.

(100 μM) and ammonium molybdate (10 μM) were added to the extracts, the PTPase activity was inhibited by greater than 97%. Importantly, these agents *increased* the apparent tyrosine kinase activity of the extracts as measured with the RR-src peptide (Table IB). These observations strongly suggest that vanadate and molybdate increased tyrosine phosphorylation in our platelet preparations and extracts by inhibiting PTPase.

Activation of Permeabilized Platelets by Vanadate and Molybdate. In separate studies, the ability of vanadate and molybdate to activate permeabilized platelets was assessed by measuring the generation of inositol phosphates and the release of [^{14}C]serotonin from dense granules or PDGF from α-granules. Vanadate and molybdate increased the formation of the inositol phosphates (Table II). Although the increases in IP_3 were small, they were comparable to those that occurred in response to 100 μM GTPγS. Moreover, like GTPγS, vanadate and molybdate increased the phosphorylation of serine/threonine residues in a 43-kDa protein (see Figure 1). Treatment of electropermeabilized platelets with a mixture of vanadate (100 μM) and molybdate (10 μM) also led to a time-dependent release of serotonin (Figure 6A) and PDGF (Figure 6B) into the incubation medium. The effect of the inhibitors on serotonin secretion was 2-fold greater than that of a maximal dose of thrombin, i.e., 10 nM (in five of six

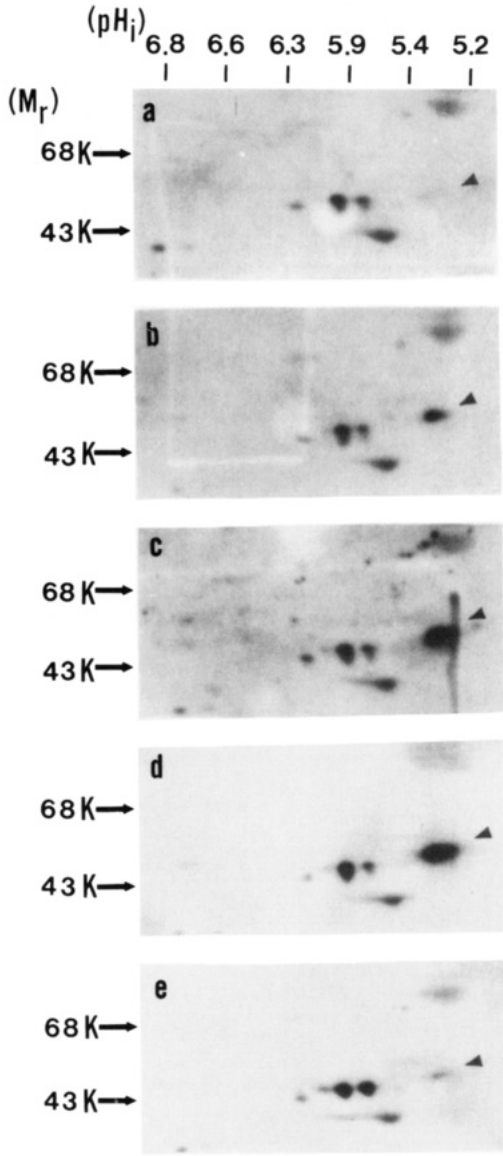

FIGURE 4: Western analysis of platelet proteins separated by using two-dimensional polyacrylamide gels. Permeabilized platelets were incubated with 100 μM vanadate and 10 μM molybdate for varying times. The reactions were quenched by rapid centrifugation at 10000*g* for 30 s and the platelets suspended in isoelectric focusing sample buffer. Two-dimensional polyacrylamide gel electrophoresis was conducted as described under Experimental Procedures, and Western analysis was conducted as described in Figure 3 with the exception that ^{125}I-protein A was used as the detection system. These panels show the autoradiogram of the nitrocellulose. The incubation period represented by each panel is as follows: panel a, 0 min; panel b, 2 min; panel c, 4 min; panel d, 8 min; panel e, 4 min. Panel e represents a Western analysis performed in the presence of an excess of phosphotyrosine.

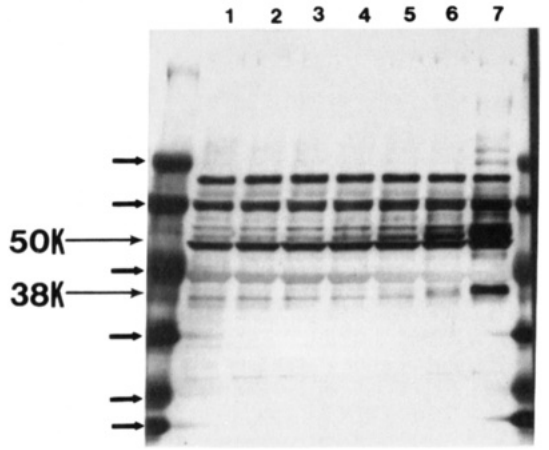

FIGURE 5: Dose response for the effect of a mixture of vanadate and molybdate on tyrosine phosphorylation. Platelets were electropermeabilized, suspended in Hepes buffer, and preincubated as described in Figure 3. The platelets were then incubated for 8 min with varying concentrations of a mixture of vanadate and molybdate. The reactions were stopped, and changes in protein tyrosine phosphorylation were assessed by using the HRP developing system as described under Experimental Procedures. Lane 1, 0.5 μM vanadate/0.05 μM molybdate; lane 2, 1.0 μM vanadate/0.1 μM molybdate; lane 3, 10 μM vanadate/1 μM molybdate; lane 4, 20 μM vanadate/2 μM molybdate; lane 5, 50 μM vanadate/5 μM molybdate; lane 6, 100 μM vanadate/10 μM molybdate; lane 7, 250 μM vanadate/25 μM molybdate. This figure is representative of two separate experiments.

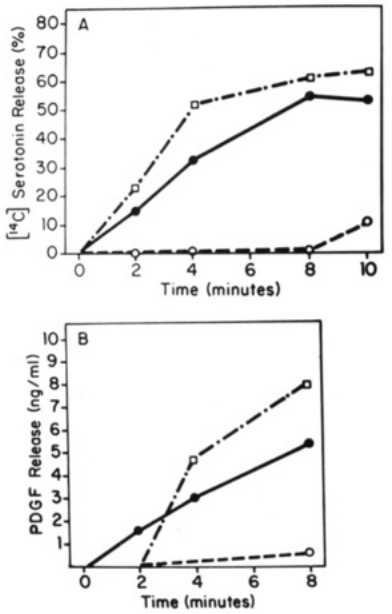

FIGURE 6: Time course of [^{14}C]serotonin and PDGF release induced by GTPγS or by a mixture of vanadate and molybdate. (A) [^{14}C]Serotonin-labeled platelets were electropermeabilized, suspended in Hepes buffer, and preincubated with ATP and $MgCl_2$, as described under Experimental Procedures. The platelets were then incubated at 25 °C with buffer alone (○), buffer + GTPγS (100 μM) (□), or buffer + a mixture of vanadate (100 μM) and molybdate (10 μM) (●). At the times indicated, aliquots were removed, and the amount of [^{14}C]serotonin released was assessed as previously described. This graph is representative of eight different experiments. (B) Electropermeabilized platelets were treated with either buffer (○), buffer + GTPγS (100 μM) (□), or buffer + a mixture of vanadate (100 μM) and molybdate (10 μM) (●). At the times indicated, aliquots were removed, and the amount of PDGF released was assessed as described under Experimental Procedures.

experiments, data not shown), but was only 75% as great as that of 100 μM GTPγS (Figure 6). Both vanadate and molybdate individually stimulated the release of serotonin in separate experiments (Figure 7), whereas the addition of a mixture of the protein serine/threonine phosphatase inhibitors (5 mM β-glycerophosphate, 5 mM *p*-nitrophenyl phosphate, and 10 mM pyrophosphate) was without effect.

Both the vanadate/molybdate mixture and GTPγS presumably acted intracellularly since no stimulation of the release of serotonin occurred upon their addition to intact platelets. Similarly, the vanadate/molybdate mixture and GTPγS (as well as thrombin) had no stimulatory effect in the absence of added ATP. All of these were effective in the presence of aspirin, which suggests that thromboxane did not mediate their actions.

Comparison of the Rate of Tyrosine Phosphorylation of the 50-kDa Protein(s) with Serotonin Release. The rela-

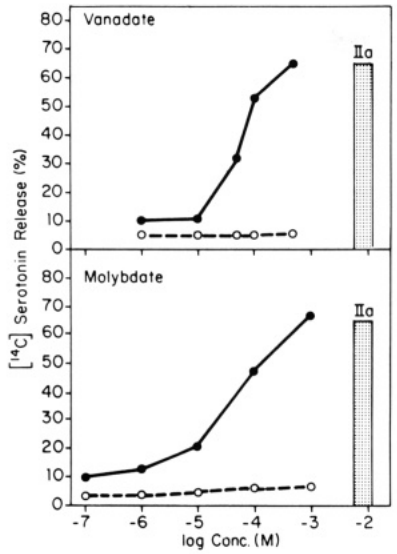

FIGURE 7: Dose response curves for the effects of vanadate and molybdate on the release of [^{14}C]serotonin. [^{14}C]Serotonin-labeled platelets were electropermeabilized, suspended in Hepes buffer, and preincubated with ATP and $MgCl_2$, as described under Experimental Procedures. The platelets were then incubated for 10 min at 25 °C with varying concentrations of either vanadate (top figure, solid symbols) or molybdate (bottom figure, solid symbols). The reactions were stopped, and the amount of serotonin released was assessed as described under Experimental Procedures. The open symbols represent the effect of either vanadate or molybdate on serotonin release from intact platelets. One unit/mL thrombin (IIa) was added to the intact platelets to show responsiveness to external agonists. Similar results were obtained in a second experiment.

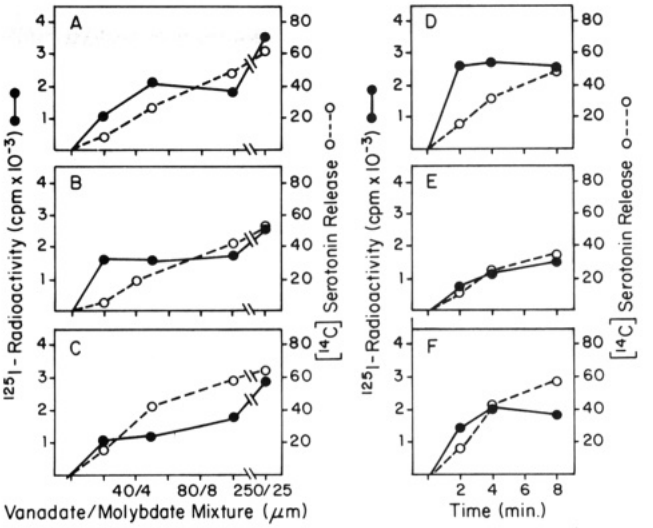

FIGURE 8: Dose response and time course for the effects of a mixture of vanadate and molybdate on tyrosine phosphorylation and [^{14}C]-serotonin secretion. [^{14}C]Serotonin-labeled platelets were electropermeabilized, suspended in Hepes buffer, and preincubated as previously described. The platelets were then incubated for 8 min with varying concentrations of a mixture of vanadate and molybdate (panels A–C) or for varying times with 100 μM vanadate and 10 μM molybdate (panels D–F). The reactions were stopped, and the amount of serotonin released or changes in protein tyrosine phosphorylation were assessed as described under Experimental Procedures. For these studies, ^{125}I-protein A was used in the Western analysis. The amount of ^{125}I-protein A associated with the 50-kDa species was assessed after it was excised from the nitrocellulose. Each panel is the result of an independent experiment.

tionship between phosphorylation of the 50-kDa protein(s) on tyrosine and serotonin release was measured as a function of both vanadate and molybdate concentration and time. Two series of reaction mixtures were used: one to assess serotonin release, the other to quantitate tyrosine phosphorylation of the 50-kDa protein(s) by Western analysis. In three separate experiments, the dose of vanadate and molybdate that was required to induce serotonin release was comparable to that required to induce phosphorylation of the 50-kDa protein(s) on tyrosyl residues (Figure 8, left panels). In three other experiments, similar rates of tyrosine phosphorylation and serotonin release were observed (Figure 8, right panels). These data raise the intriguing possibility that tyrosine phosphorylation of the 50-kDa protein may be causally linked to platelet activation.

Discussion

In the present study, the addition of vanadate and molybdate to electropermeabilized human platelets consistently caused a time- and dose-dependent increase in the content of phosphotyrosine in proteins of 50 and 38 kDa. This effect probably reflected the inhibition of PTPases and not the activation of protein-tyrosine kinases (Gresser et al., 1987). The identity and function of the two tyrosine-phosphorylated proteins remain to be established. Among many possibilities, the 50-kDa species may be the phosphotyrosyl-containing protein that Phan-Dinh-Tuy et al. (1983) observed in detergent-insoluble fractions of platelets: both proteins migrated similarly on isoelectric focusing gels. Alternatively, it could be related to the 50-kDa protein-tyrosine kinase that was recently isolated from human platelets and that undergoes autophosphorylation (Presek et al., 1988).

Surprisingly, tyrosine phosphorylation of the 50-kDa protein, which was prominently and consistently observed here, was not found in the studies of thrombin-activated platelets reported by Ferrell and Martin (1988), Golden and Brugge (1989), or Nakamura and Yamamura (1989). Ferrell and Martin (1988) demonstrated that changes in tyrosine phosphorylation occurred in three distinct temporal waves in proteins of 27, 34, 68, 70, 105, 115, and 130 kDa, whereas Golden and Brugge (1989) consistently found changes in the phosphotyrosyl content in proteins of 95–97 kDa as well as in proteins of 34, 38, 77, 83, 116, and 170 kDa. In contrast, Nakamura and Yamamura (1989) found changes in phosphotyrosyl content in only three proteins, i.e., 76, 124, and 135 kDa. The reason why the 50-kDa phosphoprotein was detected only in our studies is not yet known. The phosphorylation of the 50-kDa protein on tyrosyl residues may be extremely labile and therefore detectable only after PTPases are inhibited. Supporting this possibility, we did not detect increased phosphorylation of the 50-kDa protein in thrombin-activated platelets. Another possibility is that the difference may reflect platelet manipulations. In support of this possibility, Golden and Brugge (1989) demonstrated dissimilar phosphorylation patterns in platelets that had been isolated by using different procedures. Our isolation procedures were similar to those of Nakamura and Yamamura (1989). However, it remains possible that the 50-kDa protein is a proteolytic fragment, an artifact of electropermeabilization, although it was detected even in the presence of protease inhibitors, EGTA, and leupeptin (data not shown). A third possibility is that the difference may be related to the strategies and detection systems used. For example, the different results obtained by Ferrell and Martin (1988), Golden and Brugge (1989), and Nakamura and Yamamura (1989) using anti-phosphotyrosine immunoblot analyses could be a reflection of the antibody preparations used (Wang, 1988; Golden & Brugge, 1989). It is noteworthy in this regard that the increased tyrosine phosphorylation of the 50-kDa protein that occurred in our experiments could be detected without using a phosphotyrosine antibody. However, it is also noteworthy that we failed to

detect tyrosine phosphorylation other than in the 38- and 50-kDa proteins even when we used a phosphotyrosine antibody. As mentioned earlier, the tyrosyl phosphorylations of the 50- and 38-kDa proteins may be particularly sensitive to PTPase activities. Alternatively, the absence of tyrosine phosphorylation of other proteins may have been the consequence of our use of a permeabilized system in which small intracellular compounds were rapidly lost. In intact platelets, the phosphorylation of several proteins on tyrosyl residues appears to be dependent on aggregation events (Ferrell & Martin, 1989). New data have suggested that aggregation itself may be dependent on the formation of histamine as a second messenger (Saxena et al., 1989). Thus, if small messenger molecules, such as histamine or other unidentified compounds, trigger platelet responses that are linked to the tyrosine phosphorylation of certain platelet proteins, increased tyrosine phosphorylation of these proteins might not be detectable in a permeabilized system.

The addition of vanadate and molybdate to electropermeabilized platelets also caused changes in several events believed to be part of the activation cascade. Like GTPγS, vanadate and molybdate stimulated an increase in inositol phosphates and in serine/threonine phosphorylations, particularly involving a 43-kDa protein which is probably the "47"-kDa platelet protein substrate for protein kinase C (Tyers et al., 1988). In addition, these agents also stimulated the secretion of serotonin and PDGF. The mechanism(s) of these effects is (are) not known, but at least three possibilities can be proposed. First, as recently suggested by Paris and Pouyssegur (1987), vanadate may activate guanine nucleotide binding proteins which in turn activate the degradation of phosphoinositides. Second, tyrosine phosphorylation of the 50-kDa protein may promote the degradation of phosphoinositides by analogy with the phosphorylation of phospholipase C in response to EGF (Wahl et al., 1988). Lastly, the effects of vanadate on the platelet activation cascade and on the tyrosine phosphorylation of the 50-kDa protein may reflect separate events that may act in concert to bring about the full platelet response. Clearly, more studies will be needed to distinguish between these and other possibilities.

In conclusion, this study emphasizes the possibility that the tyrosine phosphorylation of certain platelet proteins may be tightly regulated by PTPases. It also shows that vanadate and molybdate can activate permeabilized platelets. The fact that the effects of these agents on the tyrosine phosphorylation of a 50-kDa protein and on platelet activation show a similar time and dose dependence raises the intriguing possibility that the tyrosine phosphorylation of the 50-kDa protein might be part of the platelet activation cascade.

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Registry No. PTPase, 79747-53-8; IP, 15421-51-9; IP_2, 27216-57-5; IP_3, 27121-73-9; L-Tyr, 60-18-4; serotonin, 50-67-9; inositol phosphate, 68247-19-8.

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