Activation of signal transduction in platelets by the tyrosine phosphatase inhibitor pervanadate (vanadyl hydroperoxide)

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The protein tyrosine phosphatase (PTPase) inhibitor pervanadate (vanadyl hydroperoxide) stimulated protein tyrosine phosphorylation 29-fold more than did thrombin in intact and saponin-permeabilized platelets. Increased tyrosine phosphorylation preceded, or was coincident with, a fall in PtdIns(4,5) P_2 levels, production of PtdIns(3,4) P_2 and phosphatidic acid, mobilization of intracellular Ca^{2+} , stimulation of protein kinase C-dependent protein phosphorylation, secretion of dense and α -granules, increased actin polymerization, shape change and aggregation which required fibrinogen and was mediated by increased surface expression of GPIIb-IIIa. The tyrosine kinase inhibitor RG 50864 totally prevented induction of tyrosine phosphorylation by pervanadate, as well as all other responses measured; in contrast, the inactive structural analogue, tyrphostin #1, had no effect. Dense-granule secretion induced by pervanadate required protein kinase C activity; however, aggregation and α -granule secretion were independent of protein kinase C. In saponin-permeabilized platelets pervanadate and thrombin stimulated phospholipase C activity by GTP-independent and GTP-dependent mechanisms respectively. We conclude that PTPases are important regulators of signal transduction in platelets.

INTRODUCTION

Human platelets possess very high levels of the protein tyrosine kinase pp60^{c-src}, and an unusually high proportion of protein phosphotyrosine residues under resting conditions [1]. Other PTKs of the *src* family, namely pp60^{lyn}, pp54,58^{lyn}, pp61^{hck} and pp62^{ves}, are also present [2,3]. As few as two [4], to as many as 22 [5], tyrosine-phosphorylated polypeptide bands have been reported by different groups upon immunoblotting with various anti-phosphotyrosine antibodies. The discovery that thrombin stimulates tyrosine phosphorylation [5,6] suggests a role in signal transduction in platelets, which has not yet been established. No increase in the activity of pp60^{c-src} or pp60^{lyn} has been found to accompany stimulation of platelets. Platelets also have very high protein tyrosine phosphatase (PTPase) activity [7,8], and several PTPase cDNAs have been cloned from a human megakaryoblastic cell line, including the transmembrane receptor phosphatase CD45, the soluble tyrosine phosphatases PTPase-1B and T-cell phosphatase, and a novel PTPase with similarity to band 4.1 and certain cytoskeletal proteins [8]. The specific roles of these phosphatases in platelet activation have not been defined.

Tyrosine phosphorylation has been indirectly implicated in several platelet functions, such as phospholipase C (PLC) activation [4,9], aggregation [4,9] and secretion [7,9]. Salari *et al.* [9] reported that the tyrosine kinase inhibitor erbstatin, but not genistein, blocked all responses of rabbit platelets to platelet-activating factor, i.e. tyrosine phosphorylation, Ins(1,4,5) P_3 production, protein kinase C (PKC) activation, aggregation and 5-hydroxytryptamine (5-HT) secretion. Erbstatin also inhibited secretion and aggregation by thrombin. Dhar *et al.* [4], on the contrary, found that high concentrations of genistein (0.25 and

0.4 mM) inhibited aggregation and Ins(1,4,5) P_3 production in rabbit platelets stimulated by platelet-activating factor. These studies concluded that tyrosine phosphorylation is necessary for the activation of PLC in rabbit platelets. In contrast, we observed that 1 unit of α -thrombin/ml caused substantial hydrolysis of PtdIns(4,5) P_2 and secretion of dense-granule constituents in human platelets, despite total inhibition of the stimulated tyrosine phosphorylation by the tyrosine kinase inhibitor RG 50864 (K. Pumiglia, H. Banga & M. Feinstein, unpublished work). Hence the role of tyrosine phosphorylation in the regulation of platelet PLC activity requires further clarification.

As tyrosine phosphorylation is regulated by the balance between tyrosine kinase and PTPase activities, we have employed a PTPase inhibitor to alter this balance, and thereby affect tyrosine phosphorylation in the absence of other agonists. A mixture of orthovanadate and hydrogen peroxide forms vanadyl hydroperoxide [V(⁴⁺)-OOH] [10,11], which was termed pervanadate [11]. Pervanadate is a better inhibitor of PTPase activity than orthovanadate [12,13], penetrates cells more readily than vanadate, and is a much more potent stimulator of tyrosine phosphorylation than vanadate or H₂O₂ alone in intact cells [13,14]. Inazu *et al.* [15] recently showed that pervanadate induces aggregation of platelets with only transient tyrosine phosphorylation. However, no further characterization of platelet responses to this PTPase inhibitor have been reported. In the present study we have used the tyrphostin RG 50864, a synthetic analogue of erbstatin based on a benzylidenemalononitrile nucleus which is a potent inhibitor of epidermal growth factor (EGF) receptor tyrosine kinases. We have also employed an inactive structural analogue, tyrphostin #1, to confirm the specificity of action of RG 50864 [16]. As RG 50864 acts by competing with substrate

Abbreviations used: PTPase, protein tyrosine phosphatase; RG 50864, α -cyano-3,4-dihydroxythiocinnamide; tyrphostin #1, 4-(methoxybenzylidene)malononitrile; PLC, phospholipase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CP/CK, phosphocreatine/creatine kinase; GDP[S], guanosine 5'-[β -thio]diphosphate; BAPTA-AM, bis(*O*-aminophenoxy)-ethane-*N,N',N',N'*-tetra(acetoxymethyl) ester; PVDF, poly(vinylidene difluoride); 5-HT, 5-hydroxytryptamine (serotonin); EGF, epidermal growth factor; PtdOH, phosphatidic acid.

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rather than with ATP [16], it is a more selective inhibitor than other types of tyrosine kinase inhibitors such as genistein. We have exploited this property, in conjunction with the use of pervanadate, to further investigate the putative roles of tyrosine phosphorylation in platelet signal transduction.

MATERIALS AND METHODS

Materials

RG 50864 was provided by Dr. J. Casnellie (University of Rochester, Rochester, NY, U.S.A.) and also purchased from BIOMOL Research Labs (Plymouth Meeting, PA, U.S.A.), as was tyrphostin #1. α -Thrombin (3965 units/mg; 1 unit/ml = 6.8 nM) was provided by Dr. John Fenton II (NY State Department of Health, Albany, NY, U.S.A.) and antibody PAC-1 was supplied by Dr. S. Shattil (University of Pennsylvania, Philadelphia, PA, U.S.A.). Anti-GPIIIa antibody SZ.21 was obtained from Amac (Westbrook, ME, U.S.A.). Other reagents were obtained from the listed sources: poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA, U.S.A.); ^{125}I -labelled protein A (9.8 $\mu\text{Ci}/\mu\text{g}$), [^3H]PtdIns(4,5) P_2 (7.1 Ci/mmol) and [^{14}C]5-HT (60 mCi/mmol) (NEN-DuPont, Boston, MA, U.S.A.); luciferin/luciferase reagent (Chronolog Corp., Haverton, PA, U.S.A.); 13-azaprostanoic acid (Calbiochem, La Jolla, CA, U.S.A.); BAPTA-AM (Molecular Probes, Eugene, OR, U.S.A.), phosphocreatinine phosphate (CP), creatine kinase (CK), potato apyrase, catalase (10000 units/mg; 1 unit = 1 μmol of H_2O_2 /min at 25 °C) and peptide RGDS (Arg-Gly-Asp-Ser) (Sigma, St. Louis, MO, U.S.A.).

Preparation of pervanadate

Pervanadate was prepared by mixing 1 part of 500 mM- H_2O_2 with 5 parts of 10 mM-sodium orthovanadate in modified Tyrode's solution (145 mM-NaCl, 5 mM-KCl, 5.5 mM-glucose, 0.04 mM- CaCl_2 , 1 mM- MgCl_2 , 10 mM-Hepes, pH 7.4), and incubating at 23 °C for 10 min prior to use. This stock solution was diluted into platelet suspensions to give final concentrations of 1 mM- H_2O_2 and 0.1 mM-vanadate, which produces optimal stimulation of tyrosine phosphorylation. In some experiments a high concentration of catalase (500 units/ml) was added after formation of pervanadate to rapidly remove excess H_2O_2 . Under these conditions, pervanadate was completely effective for at least 2 h after removal of H_2O_2 ; however, a slight increase in the onset time of the action of pervanadate was noticed. This most likely reflects increased decay of pervanadate in the presence of catalase, back to vanadate, H_2 and O_2 , owing to mass action [17].

Platelet preparation

Washed platelets were prepared from human platelet-rich plasma (American Red Cross, Farmington, CT, U.S.A.) as described previously [18], following treatment with 0.2 mM-aspirin for 20 min to inactivate cyclo-oxygenase. An effect of aspirin treatment was verified by the inability of platelets to aggregate in response to 10 μM -arachidonic acid after treatment, a concentration which gave complete aggregation in untreated platelets. The platelets were resuspended in modified Tyrode's solution at the indicated concentration. Platelet aggregation was measured in a Chronolog lumiaggregometer.

Total protein phosphorylation and tyrosine phosphorylation

Protein phosphorylation was measured in washed platelets (1×10^9 /ml) preincubated with [^{32}P]P_i (0.2 mCi/ml; 800 Ci/mmol) to label the metabolic ATP pool to equilibrium, as

previously described [19]. After exposure to pervanadate, phorbol 12-myristate 13-acetate (PMA) or α -thrombin, the platelets were added to an equal volume of $2 \times$ concentrated electrophoresis buffer (3% SDS, 5% β -mercaptoethanol, 10% glycerol, 60 mM-Tris, pH 6.8) plus 200 μM - Na_3VO_4 , 20 μM - Na_3MoO_4 and 10 mM-EDTA in a boiling-water bath. After SDS/PAGE, the incorporation of ^{32}P into proteins was measured in bands that were excised after location by autoradiography [18,19]. For analysis of phosphotyrosine, the platelet proteins were electrotransferred to PVDF membranes after SDS/PAGE and immunoblotted with rabbit anti-phosphotyrosine antibody and ^{125}I -labelled Protein A, and then quantified as previously described by Pumiglia *et al.* [18].

Analysis of phospholipids

Platelets were incubated with 0.75 mCi of [^{32}P]P_i/ml for 90 min to label the phospholipids. ^{32}P -phospholipids were analysed after solvent extraction of total lipids and t.l.c. as we previously described [19]. The migration of phospholipid standards was verified by staining with I_2 . PtdIns P_2 bands were extracted and hydrolysed with methylamine to yield [^{32}P]glycerolPtdIns P_2 isomers [20] which were analysed by h.p.l.c., as described by Kuchera & Rittenhouse [21]. A [^3H]GroPtdIns(4,5) P_2 standard was prepared by hydrolysis of [^3H]PtdIns(4,5) P_2 with methylamine. A [^{32}P]GroPtdIns(3,4) P_2 standard was produced by adding [γ - ^{32}P]ATP to thrombin-stimulated saponin-permeabilized platelets, and hydrolysing the PtdIns P_2 fraction (isolated by t.l.c.) to obtain a mixture of [^{32}P]GroPtdIns(4,5) P_2 and [^{32}P]GroPtdIns(3,4) P_2 , which can be separated by ion-exchange h.p.l.c. [21]. Radioactive peaks were quantified using a FLO-ONE/beta flow detector (Radiomatic Instruments and Chemical Co., Meriden, CT, U.S.A.) with IN-FLOW BD scintillant (IN/US Systems Inc., Fairfield, NJ, U.S.A.) at an eluant/scintillant ratio of 1:4 (v/v).

Actin polymerization

Platelet cytoskeletal proteins were isolated as the Triton X-100-insoluble fraction by addition of an equal volume of lysing solution, as previously described [22]. The lysing solution, modified from that which we originally used, contained 2% Triton X-100, 80 mM-KCl, 20 mM-imidazole-HCl, 20 mM-EGTA, 4 mM- NaN_3 , 20 μM -leupeptin and pepstatin A, 2 mM-phenylmethanesulphonyl fluoride, 200 μM - Na_3VO_4 and 20 μM - Na_3MoO_4 , pH 7.0. The insoluble proteins were obtained by centrifugation (15000 g, 5 min) and subjected to SDS/PAGE and stained with Coomassie Blue in order to measure actin by densitometry [22].

Surface expression of glycoprotein (GP)IIb-IIIa complex

Expression of activated GPIIb-IIIa on the platelet surface was measured by fluorescence flow cytometry using antibody PAC-1, by a modification of the method of Shattil *et al.* [23]. Washed platelets (55 μl ; 1×10^8 /ml) in modified Tyrode's solution were mixed with 5 μl of PAC-1 antibody (390 $\mu\text{g}/\text{ml}$) and 5 μl of a 6.4-fold dilution of pervanadate stock solution, and incubated for 15 min, followed by incubation with fluorescein isothiocyanate-conjugated-anti-IgM antibody for 15 min. The platelets were diluted to 0.5 ml for measurement of fluorescence in a Becton-Dickinson FACScan flow cytometer; excitation 488 nm, emission 530 nm.

Secretion of α -granule proteins, ATP and 5-HT

5-HT secretion was measured in washed platelets (1×10^9 /ml) prelabelled with 0.2 μCi of [^{14}C]5-HT/ml, as previously described [25]. Briefly, following stimulation, platelets were first fixed with 0.2 vol. of ice-cold 1 M-formaldehyde/50 mM-EDTA, followed

by centrifugation (8000 *g*, 1.5 min) to pellet the cells. Radioactivity in an aliquot of the supernatant was measured, and that present in the supernatant from unstimulated cells (5–6 % of total) was subtracted from all samples. To measure ATP secretion, 0.5 ml aliquots of washed platelets (3×10^8 /ml) were added to 25 μ l of luciferin/luciferase reagent in the lumiaggregometer. The light emission was calibrated using a range of ATP standards added to suspensions of unstimulated platelets. To measure α -granule protein secretion, washed platelets (1×10^9 /ml) were treated with stimulating agents, then sedimented for 1.5 min in a microcentrifuge at 8000 *g*. The supernatant was added to an equal volume of 2 $\times$ concentrated electrophoresis buffer in a boiling-water bath for 4 min. The proteins were separated by SDS/PAGE, stained with Coomassie Blue and quantified by densitometry. Thrombospondin identification was based on the observation by Baenzinger *et al.* [24] that this protein is the major high-molecular-mass protein found in the stimulated platelet supernatant, migrating in our system at 180 kDa. Fibrinogen was measured by monitoring the B β and τ chains, the identity of which was confirmed by Western analysis with anti-fibrinogen antibody (Calbiochem). The metabolic pool of adenine nucleotides was labelled with 1 μ M-[3 H]adenine (10 μ Ci/ml) in order to measure any possible lysis or permeabilization by pervanadate. Secretion of ATP, 5-HT or α -granule proteins induced by pervanadate was not due to permeabilization or lysis of platelets, as it was not accompanied by release of the metabolic pool of adenine nucleotides (labelled with [3 H]adenine) or cytoplasmic proteins (results not shown).

RESULTS

Effect of pervanadate on tyrosine phosphorylation

H₂O₂ (1 mM) or vanadate (0.1 mM) alone had little or no effect on tyrosine phosphorylation in platelets (Fig. 1*a*), or on any of the other platelet responses that were measured. Therefore, unless otherwise specified, only the results with pervanadate (final concentrations 1 mM-H₂O₂/0.1 mM-vanadate; see the Materials and methods sections) will be discussed. Pervanadate added to 10^9 platelets/ml increased tyrosine phosphorylation of as many as 27 protein bands at 37 °C over a period of 15 min, affecting all the same proteins as thrombin, but also a number of proteins that were not detectable when thrombin was the stimulus. The most prominent bands stimulated by pervanadate were (in kDa): 170, 143, 140, 135, 128, 118, 104, 93, 79, 65, 56, 39, 32 and 30. Tyrosine phosphorylation was observed over a concentration range of 12.5–100 μ M-vanadate, with an EC₅₀ of approx. 40 μ M in the presence of 1 mM-H₂O₂, in good agreement with the findings of Inazu *et al.* [15]. In contrast, however, we found that under the conditions we employed, tyrosine phosphorylation was not transient, but rather continued to increase over the time period measured (up to 20 min). This would be predicted, as the action of pervanadate is to inhibit PTPases (see below). Total tyrosine phosphorylation was increased 29-fold more than with thrombin (Fig. 1). The lesser effect of thrombin probably reflects, in part at least, the counter-effects of active PTPases, which pervanadate treatment inactivates. It should be noted that all responses to pervanadate occurred in aspirin-treated platelets and in the presence of ADP scavengers (2 units of apyrase/ml or CP/CK 5 mM/8 units/ml), and hence were independent of the thromboxane synthesized from arachidonic acid or secreted ADP.

At 10^9 platelets/ml, the onset and rate of tyrosine phosphorylation in intact platelets at 37 °C were slow, and did not occur when the temperature was lowered to 23 °C. We attribute this to the slow permeation of the plasma membrane by pervanadate,

since near-maximal tyrosine phosphorylation was attained within only 30–60 s at either 23 °C or 37 °C when pervanadate was added to saponin-permeabilized platelets (Fig. 1*b*).

As we previously noted with phorbol esters [26], the onset and rate of tyrosine phosphorylation, phosphatidic acid production, and aggregation in response to pervanadate increased as the concentration of platelets decreased (Fig. 2; also compare Figs. 4 and 5). This may be due to two causes: (i) the pervanadate/

Fig. 1. Tyrosine phosphorylation in intact and permeabilized platelets

Western blots of 100 μ g of protein per lane using anti-phosphotyrosine antibody and 125 I-Protein A. (a) Intact washed platelets (1×10^9 /ml); lanes: 1, untreated; 2, 1 mM-H₂O₂ for 15 min; 3, 0.1 mM-vanadate for 15 min; 4, pervanadate for 15 min; 5, 1.0 unit of thrombin/ml for 5 min. The temperature was 37 °C. Note that the exposure time for autoradiographs was optimized for pervanadate. Longer exposure times revealed many more bands stimulated by thrombin (e.g. see [18]); pp60^{c-src} is the most prominent band. Total 125 I radioactivity in protein bands: thrombin, 2309 $\pm$ 273 c.p.m. (means $\pm$ range), pervanadate, 67905 $\pm$ 7160 c.p.m. (b) Washed platelets (1×10^9 /ml) permeabilized with 25 μ g of saponin/ml in buffer described by Kuchera & Rittenhouse [21]. Free Ca²⁺ was buffered to 100 nM by EGTA. Lanes 1–6 were at 37 °C; lane 7 was at 23 °C. Lanes: 1, untreated; 2–6, pervanadate for 15 s, 30 s, 1 min, 3 min and 5 min respectively; 7, pervanadate for 5 min.

Fig. 2. Tyrosine phosphorylation by pervanadate at various concentrations of platelets

Intact washed platelets were incubated with pervanadate for 1, 3, 5 or 10 min at 37 °C, as indicated below lanes. Western blots were obtained using 70 μ g of protein/lane. Platelets/ml: (a) 3×10^8 , (b) 6×10^8 , (c) 1×10^9 .

Fig. 3. RG 50864 inhibits pervanadate-induced tyrosine phosphorylation but not PMA-mediated pleckstrin phosphorylation

RG 50864 (0–90 μM) was added to platelets ($1 \times 10^8/\text{ml}$) for 4 min, then pervanadate was added for 15 min at 37 $^\circ\text{C}$ (open symbols), or platelets were first treated with pervanadate for 15 min, and then RG 50864 was added for 5 min (closed symbols). Western blots are of 100 μg of protein/lane, with quantification as described in the Materials and methods section. Protein bands counted for radioactivity were of 79 kDa (Δ), 65 kDa ($\square$) and 39 kDa ($\circ$). Control bands contained 200–300 c.p.m., which increased to 5000–8000 c.p.m./band after pervanadate treatment. Results are means of two separate experiments $\pm$ range. Inset: ^{32}P -labelled platelets ($1 \times 10^8/\text{ml}$) were pretreated with vehicle alone (1), 90 μM -RG 50864 (2) or 1 μM -staurosporine (3) for 5 min prior to stimulation with 80 nM-PMA for 5 min. Pleckstrin (P47), the major PKC substrate in platelets, was excised and incorporated ^{32}P was counted. Under these conditions, PMA produced a 7.6-fold rise in pleckstrin ^{32}P content. Data are duplicate determinations ($\pm$ range) of one experiment, representative of data from three different experiments.

PTPase concentration ratio and (ii) the absolute amount of cellular reducing agents (or enzymes) per ml of suspension, which will accelerate the decay of pervanadate [10,17]. To this end, we have observed that exogenously added reducing agents can inhibit the actions of pervanadate, but not those of thrombin (results not shown). Therefore in all studies in this paper comparisons are made at equal concentrations of platelets.

Inhibition of pervanadate-induced tyrosine phosphorylation by the tyrosine kinase inhibitor RG 50864

The tyrosine kinase inhibitor RG 50864 slightly decreased basal tyrosine phosphorylation, and totally blocked the stimulation by pervanadate at a concentration of 90 μM ($\text{IC}_{50} = 18 \mu\text{M}$) (Fig. 3), comparable to its potency against EGF-induced growth and [^3H]thymidine incorporation in A431 and HER14 cells [16,27]. In contrast, tyrphostin #1, a structural analogue (see [16] for comparative structures) with a reported IC_{50} for EGF receptor kinase activity of more than 520 times that of RG 50864 [16], showed no inhibition of pervanadate-induced tyrosine phosphorylation. When RG 50864 was added 15 min after pervanadate, it did not alter tyrosine phosphorylation at concentrations from 90 to 450 μM (Fig. 3), demonstrating that tyrosine phosphorylation could not be reversed when PTPase activity was eliminated by pervanadate. In contrast, tyrosine phosphorylation caused by 80 nM-PMA was rapidly reversed by subsequent addition of RG 50864, implying very active PTPases in platelets not treated with pervanadate (results not shown).

Fig. 4. Effect of pervanadate on tyrosine phosphorylation, ^{32}P -labelled phosphoinositides and PtdOH

[^{32}P]-labelled washed platelets ($1 \times 10^8/\text{ml}$; aspirin-treated; +1 unit of apyrase/ml) were incubated with pervanadate for the times indicated. Lipids were extracted from 5×10^8 platelets and analysed by t.l.c. and/or h.p.l.c. (a–c). Analysis of PtdIns P_2 isomers. PtdIns P_2 bands from t.l.c. were hydrolysed and separated as described. (a) Unstimulated, (b) +pervanadate for 20 min, (c) +thrombin (1.0 unit/ml) for 5 min (included for comparison). The elution positions for standards were: a, ADP; b, [^{32}P]GroPtdIns(3,4) P_2 -, c, [^{32}P]- and [^3H]-GroPtdIns(4,5) P_2 ; d, ATP. Pervanadate decreased PtdIns(4,5) P_2 . (d) Temporal relationship between tyrosine phosphorylation ($\blacksquare$) and phospholipid changes ($\circ$, $\square$). A 100 μg sample of platelet protein was analysed by immunoblotting and bound ^{125}I was counted. Data represent changes in 79 kDa protein content; however, other proteins showed similar time courses (see Fig. 2). Data points are means $\pm$ range of duplicates; data are representative of three experiments.

Phosphoinositide metabolism and phosphatidic acid (PtdOH) production

Pervanadate, after an initial lag period of about 7 min (at 10^9 platelets/ml), caused a 20% decrease in [^{32}P]PtdIns P_2 (Fig. 4d), an initial 25% increase in [^{32}P]PtdIns P followed by a 27% decline below the initial level, and a 175% increase in [^{32}P]PtdIns. Production of [^{32}P]PtdOH is the most sensitive indicator of PLC activation in platelets because of the very large signal/noise ratio, and the fact that virtually all PtdOH is synthesized from the diacylglycerol generated by PLC [28]. [^{32}P]PtdOH was increased 16-fold (S.E.M. 0.57; $n = 3$) by pervanadate (Fig. 4d). At lower concentrations of platelets [^{32}P]PtdOH was produced more rapidly, commencing by 1 min at 1×10^8 platelets/ml (Fig. 5), in keeping with the more rapid onset of the actions of pervanadate at lower cell concentrations.

The [^{32}P]PtdOH produced most likely arose from the phosphorylation of diacylglycerol generated by the hydrolysis of phospholipids by PLC, and was not catalysed by phospholipase D (PLD) for the following reasons. (i) In intact ^{32}P -labelled platelets, the amount of [^{32}P]PtdOH produced was 10-fold greater than the entire ^{32}P -labelled phosphatidylcholine (PtdCho) +

Fig. 5. Ca^{2+} mobilization, PtdOH production and platelet shape change produced by pervanadate

Washed platelets ($1 \times 10^8/\text{ml}$; 1 unit of apyrase/ml; aspirin-treated) which had been loaded with Fura-2 ($1 \mu\text{M}$, 45 min) were used for analysis of intracellular Ca^{2+} . [^{32}P]-labelled platelets under the same conditions were used for PtdOH analysis. [Ca^{2+}], was measured as the 340 nm/380 nm excitation ratio, with fluorescence emission at 510 nm: a, thrombin (1 unit/ml); b, pervanadate. The response to pervanadate was not affected by 1 mM-EGTA present in the medium to reduce extracellular Ca^{2+} to 0.4 nM (results not shown). Incubation with BAPTA-AM ($20 \mu\text{M}$) for 15 min totally blocked Ca^{2+} mobilization (results not shown). The broken line shows [^{32}P]PtdOH analysis. [^{32}P]PtdOH increased from approx. 300 c.p.m. to 6000 c.p.m. after pervanadate treatment. The onset of shape change is manifested as decreased light transmittance in the lumiaggregometer. Pervanadate was added at the arrows. Data are representative of three experiments.

Table 1. Inhibition of thrombin-mediated, but not pervanadate-mediated, PtdOH acid formation by GDP[S]

Saponin-permeabilized platelets in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP ($500 \mu\text{M}$ in expt. a; $240 \mu\text{M}$ in expt. b; $40 \text{ mCi}/\text{mmol}$) were treated for 2 min prior to the addition of thrombin (1 unit/ml) or pervanadate with $100 \mu\text{M}$ -GDP[S]. Reactions were terminated at 5 min. Free Ca^{2+} was clamped at 100 nM by EGTA. Results are expressed as stimulated PtdOH production (c.p.m.) (resting values $\pm 100 \mu\text{M}$ -GDP[S] were subtracted). Values in parentheses indicate PtdOH production in the presence of GDP[S] as a percentage of that in its absence

Condition	Expt.	PtdOH (c.p.m.)	
		–GDP[S]	+GDP[S]
Thrombin	A	92486	14854 (16)
	B	58412	5122 (9)
Pervanadate	A	147916	138380 (94)
	B	37564	39904 (106)

phosphatidylethanolamine (PtdEtn) pool, which are the preferred substrates for PLD [28,29], and no changes in the PtdCho-PtdEtn pool were observed. (ii) The phosphoinositides, which have previously been shown not to be substrates for platelet PLD [29], would have to completely turn over once to account for the amount of [^{32}P]PtdOH produced. (iii) Pervanadate greatly stimu-

Table 2. Effect of tyrosine kinase inhibition on pervanadate-induced PtdOH production in intact platelets

[^{32}P]PtdOH production was measured in intact platelets ($1 \times 10^9/\text{ml}$) treated with pervanadate for 15 min; vehicle (dimethyl sulphoxide), $90 \mu\text{M}$ -RG 50864 or $90 \mu\text{M}$ -tyrphostin #1 were added 5 min before pervanadate. Under these conditions, RG 50864 completely inhibited pervanadate-induced tyrosine phosphorylation, but tyrphostin #1 was without effect. Values are the difference in PtdOH production compared with unstimulated platelets and are representative of data from two separate experiments; values in parentheses are % of control

Condition	PtdOH (c.p.m.)
Pervanadate	29816 (100)
Pervanadate + $90 \mu\text{M}$ -RG 50864	2807 (9)
Pervanadate + tyrphostin 1	32023 (107)

lated [^{32}P]PtdOH production in saponin-permeabilized platelets in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP (Table 1), a condition in which there was no detectable ^{32}P -labelling of PtdCho or PtdEtn, and the total [^{32}P]phosphoinositides contained only one-fifth of the ^{32}P in PtdOH. (iv) Hydrolysis of phospholipids by PLC would generate diacylglycerol (in contrast to PLD) and hence increased activation of PKC would be predicted, a response which in fact we observed (see below and Fig. 6).

Pervanadate also greatly stimulated [^{32}P]PtdIns(3,4) P_2 synthesis (Figs. 4a–4c), increasing it from $<1\%$ of the total [^{32}P]PtdIns P_2 to as much as 15% of this fraction. It should be noted that the analysis by t.l.c. (Fig. 4d) does not discriminate between these two isomers; therefore the hydrolysis of the PtdIns(4,5) P_2 isomer is predicted to be about 34% of the basal levels. This is confirmed by h.p.l.c. analysis. PtdIns 3-kinase was previously found to associate with pp60^{c-src} and p59^{lyn} in thrombin-stimulated platelets [30], implying a role for tyrosine phosphorylation in the regulation of the PtdIns 3-kinase. We found that [^{32}P]PtdIns(3,4) P_2 production was blocked by RG 50864 (but not by the inactive structural analogue tyrphostin-1) implicating tyrosine phosphorylation, but also by $1 \mu\text{M}$ -staurosporine, consistent with the data of King *et al.* [31] that indicated a role for PKC in the stimulation of the PtdIns 3-kinase. Further studies will be necessary to elucidate the relationship between these two pathways in regulating PtdIns(3,4) P_2 metabolism.

Regulation of pervanadate-mediated PLC activation

The role of G-proteins in the activation of PLC by pervanadate was assessed by monitoring the production of [^{32}P]PtdOH in saponin-permeabilized platelets using [$\gamma\text{-}^{32}\text{P}$]ATP. Thrombin and pervanadate both stimulated formation of [^{32}P]PtdOH. Guanosine 5-[β -thio]diphosphate (GDP[S]; $100 \mu\text{M}$), which prevents G-protein activation, blocked the production of [^{32}P]PtdOH stimulated by thrombin (consistent with the studies of Brass *et al.* [32]), but had no effect on [^{32}P]PtdOH production induced by pervanadate (Table 1). Additionally, the decrease in PtdIns(4,5) P_2 level and production of PtdOH were dependent on tyrosine phosphorylation, as these effects were completely inhibited by $90 \mu\text{M}$ RG 50864 (Table 2). The effects of RG 50864 are unlikely to be the result of direct inhibition of either diacylglycerol kinase or PLC, for the following reasons: (i) tyrphostin-1, a structural analogue which does not inhibit tyrosine phosphorylation, had no effect on the lipid response to pervanadate (Table 2), (ii) RG 50864 did not inhibit [^{32}P]PtdOH formation from 1,2-dioctanoylglycerol (DiC₈) added to ^{32}P -loaded platelets (results

not shown), and (iii) RG 50864 has been shown not to directly inhibit PLC- γ 1 [33].

Ca²⁺ mobilization

Pervanadate caused a rise in intracellular Ca²⁺, to a lesser extent than did thrombin, as measured with Fura-2. This occurred with EGTA in the extracellular medium, indicating mobilization of an intracellular source of Ca²⁺. Fig. 5 shows that the rise in Ca²⁺ slightly preceded PtdOH production, as would be expected if Ins(1,4,5)P₃ arising from the hydrolysis of PtdIns(4,5)P₂ was the stimulus for Ca²⁺ release. As vanadate was previously reported to inhibit Ca²⁺-ATPase activity [34], we considered that pervanadate might release Ca²⁺ by inhibiting the intracellular Ca²⁺ pump. However, pervanadate had no effect on ATP-dependent uptake of ⁴⁵Ca²⁺ by a microsomal membrane fraction isolated as previously described [35] (results not shown), indicating that pervanadate was stimulating release rather than inhibiting uptake to produce a rise in intracellular Ca²⁺.

Protein phosphorylation

Pervanadate stimulated protein phosphorylation to 75% of the maximum produced by PMA (results not shown). The major phosphorylated protein in both cases was pleckstrin (47 kDa), the principal substrate for PKC in platelets. Staurosporine (1 μ M/10⁹ platelets per ml) totally abolished pleckstrin phosphorylation by pervanadate (Fig. 6) or by a maximally effective concentration of PMA (Fig. 3). Although staurosporine can inhibit some tyrosine kinases [36], we obtained quite selective inhibition of PKC by careful control of the staurosporine/platelet concentration ratio, i.e. staurosporine caused only slight ($\leq 20\%$) inhibition of the total tyrosine phosphorylation caused by pervanadate, with no change in the pattern of tyrosine phosphorylation (results not shown). RG 50864, but not tyrphostin #1, also abolished pleckstrin phosphorylation induced by pervanadate (Fig. 6). RG 50864, however, had no effect on pleckstrin phosphorylation by PMA (Fig. 3); hence it did not directly inhibit PKC. From these data, we conclude the following. (i) Protein phosphorylation seen with pervanadate was not the result of inhibition of Ser/Thr phosphatases, as this mode of phosphorylation would not be inhibited by RG 50864. (ii) Based on the sensitivity to RG 50864 and the identical time courses, pervanadate-induced protein phosphorylation must occur downstream from tyrosine phosphorylation, probably due to the diacylglycerol resulting from activation of PLC. (iii) Tyrosine phosphorylation caused by pervanadate is largely independent of PKC, as 1 μ M-staurosporine inhibited completely the PKC phosphorylation while the tyrosine phosphorylation was largely intact. However, at this time it is not possible to distinguish whether the slight inhibition by 1 μ M-staurosporine arises from direct inhibition of tyrosine kinases or inhibition of PKC, which is known to stimulate platelet tyrosine phosphorylation [6].

Secretion of dense-granule and α -granule constituents

Pervanadate caused secretion of [¹⁴C]5-HT (Fig. 6b) with a time course similar to that of PtdOH production and pleckstrin phosphorylation (see Figs. 4d and 6a). Secretion was totally blocked by 90 μ M-RG 50864, but also by 1 μ M-staurosporine (Fig. 6b), suggesting an obligatory role for PKC. As the experiments were conducted in the presence of 5 μ M-imipramine to block 5-HT re-uptake, the increase in extracellular 5-HT must be the result of degranulation rather than effects on amine uptake. Additionally, experiments measuring the secretion of ADP gave identical results (not shown). Pervanadate also stimulated the secretion of the α -granule proteins thrombospondin and fibrinogen (B β and τ chains) 7.0–8.3-fold. The secretion of α -granule

Fig. 6. Effect of RG 50864 and staurosporine on pleckstrin phosphorylation and dense-granule secretion induced by pervanadate

(a) Time course of pleckstrin phosphorylation induced by pervanadate in washed platelets (1 $\times 10^9$ /ml). (b) Time course of dense-granule secretion, as monitored by [¹⁴C]5-HT, in washed platelets (1 $\times 10^9$ /ml): ○, pervanadate alone; ■, preincubation for 5 min with 90 μ M-RG 50864 and then pervanadate; ▲, 5 min preincubation with 1 μ M-staurosporine and then pervanadate. At 20 min, [¹⁴C]5-HT released by pervanadate represented 56% of the total, and 90% of the maximum released by thrombin (1 unit/ml). Data are duplicate determinations ($\pm$ range) for one experiment and are representative of two separate experiments.

proteins was totally blocked by RG 50864; however, in contrast to dense-granule secretion, α -granule secretion was only modestly inhibited by 1 μ M-staurosporine ($26 \pm 2\%$; mean $\pm$ range; $n = 2$). EGTA and RGDS had no effect on the secretion of dense or α -granules (results not shown), indicating these were not aggregation-dependent responses. However, addition of BAPTA-AM (20 μ M) did substantially (70%) decrease α -granule secretion, indicating an important role for Ca²⁺ in mediating this response.

Actin polymerization and GPIIb-IIIa expression

Pervanadate increased the Triton X-100-insoluble cytoskeletal actin by 2.45-fold within 3 min, compared with a 3.45-fold increase induced by 1 unit of thrombin/ml (at 3×10^8 platelets/ml). Pervanadate also increased surface expression of the fibrinogen receptor, activated GPIIb-IIIa, as measured by binding of PAC-1 antibody to platelets. This effect of pervanadate was not inhibited by 1 μ M-staurosporine, despite total inhibition of pleckstrin phosphorylation at this concentration, suggesting that PKC was not necessary for GPIIb-IIIa expression in response to pervanadate. The effect of RG 50864 could not be

Fig. 7. Platelet aggregation and activation of GPIIb-IIIa by pervanadate

(a) Washed platelets (3×10^8 /ml) were treated with pervanadate $\pm$ inhibitors: a, pervanadate alone; b, staurosporine (1μ M) for 4 min; c, + 1 mM-EGTA; d, + 0.7 mM-RGDS; e, 20 μ M-BAPTA-AM for 15 min. (b) Binding of PAC-1 antibody to platelets in platelet-rich plasma (aspirin-treated) was measured by fluorescence cell cytometry. The abscissa is the relative fluorescence intensity on a log scale, and the ordinate is the number of cells (10000 platelets were analysed in each experiment). a, Unstimulated platelets; b, + pervanadate for 15 min; c, pervanadate + staurosporine at 1μ M (4 min pretreatment). (c) Dose-response for RG 50864-mediated inhibition of pervanadate-induced aggregation. Washed platelets (3×10^8 /ml) were pretreated for 4 min with various concentrations of RG 50864 as indicated in μ M followed by the addition of pervanadate. Stirring was begun 15 min later as indicated by time zero on the tracing. Compare with dose-response of tyrosine phosphorylation (Fig. 3). All experiments are representative of results obtained from at least three different donors.

assessed because its absorbance spectrum overlapped that of the fluorescein-labelled second antibody.

Platelet aggregation

Pervanadate induced platelet aggregation, with a lag period and time course dependent on the concentration of platelets, which was consistent with rate of tyrosine phosphorylation under the same conditions. The lag times decreased from 6 min at 10^9 platelets/ml to 2.2 min at 3×10^8 platelets/ml, and to about 1 min at 1×10^8 platelets/ml (compare Figs. 5 and 7a). Neither the extent of platelet aggregation nor tyrosine phosphorylation in response to pervanadate were affected by aspirin plus ADP scavengers (CP/CK, apyrase), or the thromboxane A_2 receptor antagonist 13-azaprostanoic acid. Aggregation caused by pervanadate and thrombin were similar in the following respects. (i) Both were blocked by loading platelets with BAPTA-AM, which releases the Ca^{2+} chelator BAPTA intracellularly and blocks the rise in Ca^{2+} (Fig. 7a). (ii) Both were blocked by the peptide RGDS or by chelation of extracellular Ca^{2+} with EGTA, which interfere with the function of the fibrinogen receptor GPIIb-IIIa (Fig. 7a). (iii) The inhibition by RGDS of pervanadate-mediated aggregation was reversed by 1 mg of fibrinogen/ml, suggesting that the primary event in aggregation was a GPIIb-IIIa-fibrinogen interaction. (iv) The rate of aggregation was 50% inhibited by the anti-GPIIIa antibody SZ.21 (results not shown). On the other hand, pervanadate-induced tyrosine phosphorylation was independent of aggregation, as it was not affected by RGDS or EGTA (results not shown).

RG 50864 inhibited tyrosine phosphorylation and pervanadate-induced aggregation over the same concentration range (Fig. 7c, compare with Fig. 3). In contrast, tyrphostin #1, which has no effect on tyrosine phosphorylation, was without effect on aggregation. Stirring of platelets was required for aggregation but not for tyrosine phosphorylation. This behaviour was used to explore the mechanism of action of RG 50864. When unstirred platelets were exposed to pervanadate + 90 μ M-RG 50864 for 15 min, followed by stirring, aggregation and tyrosine

phosphorylation were totally blocked (Fig. 7c). However, if platelets were incubated with pervanadate for 15 min without stirring, followed by addition of RG 50864 with immediate stirring, the tyrphostin had little or no effect on either tyrosine phosphorylation (Fig. 3) or aggregation (results not shown). Thus RG 50864 does not appear to have an extracellular effect; nor did it block aggregation when added after tyrosine phosphorylation had already occurred and could not be reversed. Moreover, although 1.0 mg of fibrinogen/ml in the medium effectively reversed inhibition by RGDS, it did not overcome inhibition of aggregation by RG 50864. This finding indicates that, under our experimental conditions, RG 50864 did not inhibit aggregation by competing with fibrinogen for GPIIb-IIIa binding, nor solely by inhibiting the release of fibrinogen from platelet α -granules.

DISCUSSION

These studies show the PTPase inhibitor pervanadate to be the most powerful promoter of tyrosine phosphorylation in human platelets so far investigated, greatly exceeding the effect of thrombin. The tyrosine phosphorylation was independent of various other factors involved in signal transduction processes, i.e. it did not require elevated intracellular Ca^{2+} , extracellular Ca^{2+} , activation of PKC, thromboxane A_2 production, aggregation, or secretion of dense-granule or α -granule constituents. On the other hand, stimulation of tyrosine phosphorylation was totally blocked by the tyrosine kinase inhibitor RG 50864, with an IC_{50} equivalent to that for its inhibition of the EGF receptor tyrosine kinase activity in intact cells [27]. An inactive structural analogue, tyrphostin #1, had no effect on tyrosine phosphorylation, even at 90 μ M. Tyrosine phosphorylation by pervanadate was not reversed by RG 50864 when the latter was added after inactivation of PTPases by pervanadate. From these results, it is evident that the inhibition of PTPases by pervanadate was the cause of increased tyrosine phosphorylation.

The very rapid and extensive effect of pervanadate in permeabilized platelets demonstrates that PTPases must normally exert

very tight control over tyrosine phosphorylation. Thus one potential mechanism by which agonists such as thrombin may stimulate tyrosine phosphorylation of proteins is by inhibition of PTPases that oppose constitutively active tyrosine kinases. This type of regulation would involve a substantial investment of cellular energy because of the futile cycling of phosphorylation/dephosphorylation reactions in the steady state. Another possible mechanism is by direct or indirect stimulation of tyrosine kinases. Vanadate and pervanadate do not directly stimulate purified insulin or EGF receptors [11,37]. However, by inactivating PTPases, pervanadate may indirectly stimulate some tyrosine kinases by permitting unrestrained autophosphorylation of activating Tyr sites on the kinases, such as the Tyr-1150 domain of the insulin receptor [38] and the Tyr-416 site of pp60^{c-src} kinase [39].

We have shown that pervanadate can stimulate virtually all platelet responses that are commonly elicited by strong agonists such as thrombin, i.e. PtdIns(4,5) P_2 hydrolysis, synthesis of PtdOH and PtdIns(3,4) P_2 , activation of PKC, internal Ca^{2+} mobilization, actin polymerization, dense-granule and α -granule secretion, GPIIb-IIIa activation and aggregation. Moreover, as with high concentrations of thrombin, these effects are independent of any secondary mediators, such as ADP or thromboxane A_2 . We conclude that all responses to pervanadate are attributable to the enhanced tyrosine phosphorylation, for several reasons. (i) Tyrosine phosphorylation precedes, or is coincident with, all the responses measured when care is taken to assess them under exactly the same conditions. (ii) The PTK inhibitor RG 50864 blocked all biochemical and functional responses that could be tested, in a dose range consistent with its inhibition of tyrosine phosphorylation. (iii) The inactive structural analogue tyrphostin-1 had no effect on tyrosine phosphorylation or any other responses to pervanadate.

Although all responses to pervanadate require tyrosine phosphorylation, some of them are ultimately mediated by the downstream activation of PKC, e.g. pleckstrin phosphorylation and dense-granule secretion. In these respects the responses to pervanadate resemble those to thrombin. Tyrosine phosphorylation in platelets was previously implicated in aggregation and the secretion of 5-HT [7,9]. However, we found that dense-granule secretion elicited by 1 unit of α -thrombin/ml and 80 nM-PMA can occur despite inhibition of tyrosine phosphorylation by RG 50864 (K. Pumiglia, H. Banga & M. Feinstein, unpublished work), but instead requires activation of PKC, in accordance with the findings of Watson *et al.* [40]. Dense-granule secretion caused by pervanadate likewise requires PKC activity. From these studies, it is not possible to rule out a modulatory role for tyrosine phosphorylation, which seems possible given the reported existence of pp60^{c-src} in the secretory granules of adrenal chromaffin cells [41] as well as the dense granules (but not the α -granules) of human platelets [42]. However, it seems clear that elevation of tyrosine phosphorylation above basal levels is neither necessary nor sufficient for dense-granule secretion.

In striking contrast to dense-granule secretion, α -granule secretion was little affected by preincubation with 1 μ M-staurosporine. In fact, the inhibition by staurosporine was only marginally greater than its slight effect on tyrosine phosphorylation (26% versus $\leq 20\%$), indicating no absolute requirement for PKC activation in α -granule secretion. This process was inhibited by tyrphostin RG 50864, indicating an obligatory role for tyrosine phosphorylation. At this time, however, it is unclear whether tyrosine phosphorylation is directly affecting this process or whether the effect is the consequence of increased Ca^{2+} resulting from PLC activation. In fact, we have found that preincubation of platelets with BAPTA-AM, in order to prevent a rise in intracellular Ca^{2+} , substantially inhibited pervanadate-

induced secretion. The finding by Lerea *et al.* [7] that secretion of platelet-derived growth factor increases over a similar time course and dose-response as tyrosine phosphorylation in electro-permeabilized platelets, in which the Ca^{2+} was clamped, argues in favour of a role for tyrosine phosphorylation. Interestingly, preliminary studies in our laboratory found that inhibiting tyrosine phosphorylation with RG 50864 partially blocked α -granule secretion in response to either thrombin or PMA (L. F. Lau & M. B. Feinstein, unpublished work). Taken collectively, these data suggest that a number of signals may converge to regulate α -granule secretion; hence further experimentation will be required to discern the role, if any, tyrosine phosphorylation has in this process.

Aggregation caused by pervanadate also resembles that caused by thrombin and other agonists, as it is accompanied by actin polymerization and is mediated by GPIIb-IIIa. For example, aggregation (i) requires extracellular Ca^{2+} , (ii) is accompanied by increased surface expression of activated GPIIb-IIIa (PAC-1 binding), and (iii) is inhibited by RGDS and a monoclonal antibody to GPIIIa. However, in contrast to dense-granule secretion, GPIIb-IIIa expression and platelet aggregation induced by pervanadate did not require protein phosphorylation mediated by PKC, as they were not prevented by staurosporine which completely inhibited PKC. Rather, tyrosine phosphorylation is a necessary event in pervanadate-mediated aggregation, as pretreatment with RG 50864 completely abolished this response in a dose-dependent manner closely corresponding to its inhibition of tyrosine phosphorylation. The structurally related tyrphostin #1 had no effect on pervanadate-induced aggregation. The effect of tyrphostin RG 50864 was not solely the result of decreased fibrinogen secretion due to effects on α -granule secretion, as fibrinogen added back to the medium did not restore aggregation. Similarly, we have found that aggregation induced by thrombin and PMA is blocked by RG 50864 (L. F. Lau & M. Feinstein, unpublished work). As recent experiments have determined aggregation to be mediated predominantly by interactions between activated GPIIb-IIIa and fibrinogen [43], these results raise the intriguing possibility that tyrosine phosphorylation may regulate the activation or surface expression of GPIIb-IIIa. Although GPIIb and IIIa can be tyrosine phosphorylated by pp60^{c-src} *in vitro* [44,45], this has not been found in intact platelets [46]. The role of tyrosine phosphorylation is therefore not established. It is clear, however, that tyrosine phosphorylation is not sufficient for aggregation, as BAPTA-AM totally abolishes platelet aggregation and partially inhibits GPIIb-IIIa activation by pervanadate.

PLC is a likely target through which pervanadate initiates the platelet responses via tyrosine phosphorylation, since it stimulates the breakdown of PtdIns(4,5) P_2 , which would account for the subsequent downstream signals that activate PKC and release internal Ca^{2+} . Furthermore, tyrosine phosphorylation of a number of proteins precedes the stimulation of phosphoinositide metabolism, and both are blocked by RG 50864 but not by tyrphostin #1. In saponin-permeabilized platelets we could distinguish a pervanadate-stimulated pathway to activate PLC from a thrombin/GTP-dependent pathway by their marked differential sensitivities to blockade by GDP[S]. The PLCs that are linked to G-proteins in other cells are not regulated by tyrosine phosphorylation [47,48]. Moreover, in several cell types stimulation by agonists with receptors linked to G-proteins [47,48], or by A1F₄ [49] did not phosphorylate PLC- γ 1 on tyrosine.

Although the thrombin [50] and thromboxane [51] receptors are members of the G-protein receptor family, and platelets contain PLC activity which is controlled by G-proteins [32,35], both agonists also stimulate tyrosine phosphorylation, which

could lead to activation of a tyrosine phosphorylation-dependent PLC. Platelets contain cytosolic PLC- γ 2 [52], which can be tyrosine-phosphorylated [53] and is therefore a potential target for regulation by tyrosine kinases/phosphatases. The more widely studied isoform, PLC- γ 1, is stimulated by activated growth factor receptors with protein tyrosine kinase activity [53–55]. To this end, it is important to note that pervanadate resulted in tyrosine phosphorylation of several proteins in the molecular mass range of PLC- γ 2 (135–150 kDa). Additionally, previous studies utilizing Rat-2 cells overexpressing PLC- γ 1 have shown increased inositol phosphate production in response to 1 unit of thrombin/ml compared with normal Rat-2 cells. No increase, however, was observed for the G-protein activator AIF_4^- . These studies suggest the presence of a G-protein-independent pathway by which thrombin may activate PLC-isoforms [53]. Our findings provide evidence for the existence of a tyrosine-phosphorylation-dependent pathway to activate PLC in platelets, and suggest that it and the G-protein-regulated pathway are independent, most likely regulating distinct species of PLCs.

In conclusion, the responses to pervanadate reveal a dominant role of PTPases in regulating tyrosine phosphorylation in platelets, and suggest that inhibition of PTPases may be a step in platelet signal transduction. Our results also support a role for tyrosine phosphorylation in certain signal transduction pathways in platelets, but not in dense-granule secretion. Critical areas for further investigation concern the mechanism(s) and involvement of PTPases in agonist-mediated tyrosine phosphorylation, and the role of tyrosine phosphorylation in a non-G-protein-dependent pathway for activation of PLC and its relationship to receptor-mediated signalling.

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