Protein tyrosine phosphatase activity modulation by endothelin-1 in rabbit platelets

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Abstract Protein tyrosine phosphorylation, modulated by the rate of both protein tyrosine kinase and protein tyrosine phosphatase activities, is critical for cellular signal transduction cascades. We report that endothelin-1 stimulation of rabbit platelets resulted in a dose- and time-dependent tyrosine phosphorylation of four groups of proteins in the molecular mass ranges of 50, 60, 70–100 and 100–200 kDa and that one of these corresponds to focal adhesion kinase. This effect is also related to the approximately 60% decrease in protein tyrosine phosphatase activity. Moreover, this inhibited activity was less sensitive to orthovanadate. In the presence of forskolin that increases the cAMP level a dose-dependent inhibition of the endothelin-stimulated tyrosine phosphorylation of different protein substrates and a correlation with an increase in the protein tyrosine phosphatase activity (11.6-fold compared to control) have been found. Further studies by immunoblotting of immunoprecipitated soluble fraction with anti-protein tyrosine phosphatase-1C from endothelin-stimulated platelets have demonstrated that the tyrosine phosphorylation of platelet protein tyrosine phosphatase-1C is correlated with the decrease in its phosphatase activity. As a consequence, modulation and regulation by endothelin-1 in rabbit platelets can be proposed through a cAMP-dependent pathway and a tyrosine phosphorylation process that may affect some relevant proteins such as focal adhesion kinase.

Key words: Endothelin-1; Protein tyrosine phosphatase; Focal adhesion kinase protein; Rabbit platelet

1. Introduction

The importance of protein tyrosine phosphorylation in the control of cellular processes such as growth, differentiation or transformation, and cytoskeletal integrity, has been well established [1,2]. The overall cellular phosphotyrosine levels are controlled by the dynamic balance of the action of both protein tyrosine kinases that catalyze phosphorylation and protein tyrosine phosphatases (PTP) which are responsible for dephosphorylation of tyrosyl residues [3,4]. The coordination of cellular phosphorylation/dephosphorylation events, i.e. the selection of the proper substrates and the coordination of the moments for protein tyrosine kinases and PTP action, is mainly conditioned by the restricted subcellular distribution of these two activities through their subcellular compartmentalization [5] or their activators [6].

Protein tyrosine phosphatases have no structural homology with the Ser/Thr-protein phosphatases and are divided into two families that include transmembrane receptor-like enzymes and intracellular proteins (non-receptor-like enzymes) [7,8]. These proteins that hydrolyze phosphotyrosyl groups have been involved in several cellular functions like tumor suppression [9], cytoskeletal reorganization [10], development and differentiation [11,12], mitotic induction [13] or growth factor [14] signaling pathways.

Previous studies have described the presence of PTPs in platelets. Thus, the membrane PTP of 53 kDa [15] or PTP-1B, a calpain substrate in thrombin-stimulated platelets, has been described [16]. In addition, two other PTPs were studied in platelets, the 105 kDa PTPH1 [17] and SH-PTP1 or PTP-1C which is predominantly expressed in hematopoietic cells and contains two Src homology-2 domains (SH2) [18]. Particularly interesting for platelet function is the PTP-1C, a critical enzyme associated with the cytoskeleton of thrombin-stimulated platelets [19].

The effect of endothelin-1 on the PTP activity has been studied in this paper, based on the integration of PTPs and protein tyrosine kinases activities in the control of cell function by their coordinated effect at the time of controlling tyrosine phosphorylation-dependent signaling pathways.

Endothelin-1 is produced by endothelial cells [20] and has some effect in many physiological systems through signal transduction pathways such as phospholipase C activation, cytosolic-free Ca^{2+} levels elevation and protein kinase C activation [21].

As we have previously demonstrated that endothelin-1 has a direct effect on the stimulation of several tyrosine phosphorylated proteins in rabbit platelets and that there is a novel pathway for platelet signal transduction in which the cAMP level could be involved in the endothelin effect on tyrosine phosphorylation [22]. In the present study we have evaluated the effect of endothelin-1 on PTP activity in order to establish the possible coordinated action of protein tyrosine phosphatases, in particular PTP-1C and kinases in rabbit platelets. The relationship between this effect and the modulation, by tyrosine phosphorylation, of some relevant proteins such as the focal adhesion kinase protein (FAK), has been also studied.

2. Materials and methods

2.1. Materials

Endothelin-1, forskolin, sodium orthovanadate and O-phosphotyrosine were purchased from Sigma. Nitrocellulose membranes were obtained from Renner GmbH. Monoclonal anti-phosphotyrosine (PY20), anti-PTP-1C and anti-FAK antibodies were from Affiniti Research Products Ltd. Goat anti-(mouse IgG)-horseradish-peroxidase conjugate was purchased from Nordic Immunology. Enhanced chem-

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iluminescence (ECL) system was acquired from Amersham International. Protein A–Sepharose was from Pharmacia Biotech Inc.

2.2. Isolation of rabbit platelets

Rabbit platelets were prepared as described earlier [23] from blood collected in 3.2% trisodium citrate. Platelets were suspended in HEPES–Tyrode's solution (10 mM N-2-hydroxyethylpi-perazin-N'-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 0.5 mM Na$_2$HPO$_4$, 5 mM glucose and 12 mM NaHCO$_3$, pH 7.4). The platelet number was estimated through the protein concentration determined by the Bradford method [24] using Bio-Rad protein assay reagent and bovine serum albumin as standard.

2.3. Stimulation of platelets

One hundred thirty micrograms of platelet protein ($\approx 6.9 \times 10^7$ platelets) suspended in Tyrode's buffer, pH 7.4, was stimulated by incubation with 2 μM endothelin-1 for 2 min in the absence or presence of the appropriate concentrations of forskolin. Platelets were preincubated for 3 min in the presence of forskolin, prior to stimulation with endothelin-1. At the end of each stimulation period, incubations were stopped at 4°C and centrifuged at 15 000×g for 15 min. The resulting platelets pellet was washed with Tyrode's buffer, pH 7.4, and resuspended in buffer containing 10 mM PIPES, pH 6.5, 0.2 mM EDTA, 145 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 4.8 μg/ml leupeptin and 1 μg/ml aprotinin. The suspension was frozen 3 times in liquid nitrogen. Protein concentration of this platelet suspension ranged from 1 to 2 mg/ml. This platelet preparation was processed in PAGE-SDS or subjected to PTP activity quantification.

2.4. Protein tyrosine phosphatase activity

PTP activity was assayed at 37°C by slight modification of the methods of Mustelin [25] and Smilowitz [26] in a reaction mixture (final volume: 250 μl) containing 100 mM sodium acetate buffer, pH 6.0, 10–20 μg freshly thawed platelet protein, 10 mM O-phosphotyrosine as substrate, and 0.2% Triton X-100. The reaction was started by adding the substrate dissolved in the same sodium acetate buffer, and stopped by adding an equal volume of 1 M NaOH. The tyrosine produced was measured spectrophotometrically [27] by using $\Sigma_{293.5} = 2330$ M^{-1} cm^{-1}, all in alkaline solution. The endogenous tyrosine was estimated in the absence of substrate.

2.5. Immunoprecipitation and immunoblotting

For immunoprecipitation experiments platelets were prepared and stimulated as described above with 2 μM endothelin-1 for 30 s, and the reaction stopped by platelets lysis in 50 mM HEPES, pH 7.2 buffer, supplemented with 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 2 mM EDTA, 10 mM sodium molybdate, 2 μg/ml aprotinin, 40 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitations were performed by incubation of the samples (≈ 100 μg of protein), redissolved in one volume of solubilization buffer (40 mM Tris-HCl, pH 7.5, 2% Triton X-100, 1.8% NaCl, 4 μg/ml aprotinin and 40 μg/ml leupeptin), with 10 μg of mouse primary monoclonal antibody or non-immune control serum, at 37°C for 1 h and 3 h at 4°C. Immune complexes were collected with Protein A–Sepharose, diluted 1:1 in PBS (10 mM sodium phosphate, pH 7.5, 140 mM NaCl) at 4°C for 2 h. The beads were washed once with cold buffers (10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 2% Triton X-100; 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.1% Triton X-100 and 10 mM Tris-HCl, pH 8.0, 0.05% Triton X-100), dissolved by boiling in SDS Laemmli sample buffer [28], and subjected to standard SDS-PAGE on 12% polyacrylamide gels. Proteins were then transferred to nitrocellulose paper by a wet blotting method (overnight at 200 mA). The non-specific binding was blocked with 1% BSA in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20, and the proteins were probed with anti-phosphotyrosine, anti-PTP-1C and anti-FAK monoclonal antibodies and detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's protocol. The immunoprecipitated PTP activity was measured, as described above, in the immune complexes redissolved in 20 μl of sodium acetate buffer.

3. Results and discussion

As protein tyrosine kinases have been related to intracellu-

lar signaling enzymes and to the activation of some mechanisms of signaling cascades, our results attempt to further define the possible effect of endothelin-1 on the early events of the signal transduction pathways in rabbit platelets, such as the phosphorylation of tyrosine residues. Using monoclonal anti-phosphotyrosine antibody, the effect of endothelin-1 on the phosphorylation of protein tyrosine residues was studied on isolated rabbit platelets. Treatment of the platelets with 2 μM endothelin-1 for 1 min caused a rapid tyrosine phosphorylation affecting those proteins in the molecular mass range of 50, 60, 70–100 and 100–200 kDa (Fig. 1A). Tyrosine phosphorylation in stimulated-platelets was at its highest level in the 60 kDa and 100–200 kDa proteins whereas in the 50 kDa and 70–100 kDa proteins the effect was moderate (Fig. 1B). By in vitro determination of total PTP activity in the
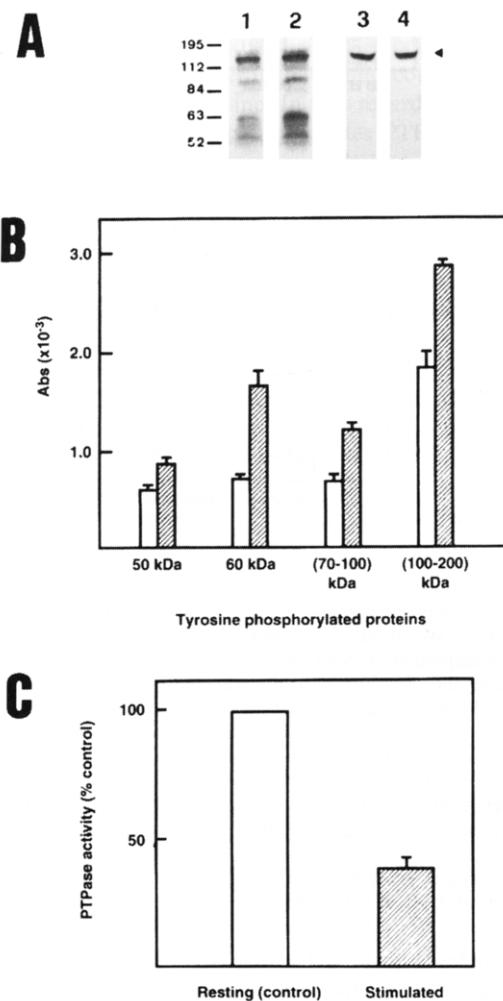

Fig. 1. Endothelin-1 induction of tyrosine phosphorylation and effect on protein tyrosine phosphatase. Platelets were stimulated with endothelin-1, as described under Section 2. A: Western blot analysis of rabbit platelet proteins on 12% SDS-PAGE, immunoblotted with anti-phosphotyrosine antibody (PY20) (lanes 1 and 2) and with anti-FAK antibody (lanes 3 and 4). Arrow indicates FAK protein, lanes 2 and 4 endothelin-stimulated platelets, and lanes 1 and 3 resting platelets. B: Tyrosine phosphorylation level in the different molecular mass ranges of proteins in stimulated platelets (■) compared to resting platelets (control) (□). C: Effect of endothelin-1 on total PTP activity (■) (0.09 U/mg protein) with respect to resting platelets (□) (0.22 U/mg protein). PTP activity was measured as described under Section 2 using 20 μg platelet proteins at 37°C for 25 min. All results represent the mean ± SE of four separate experiments.
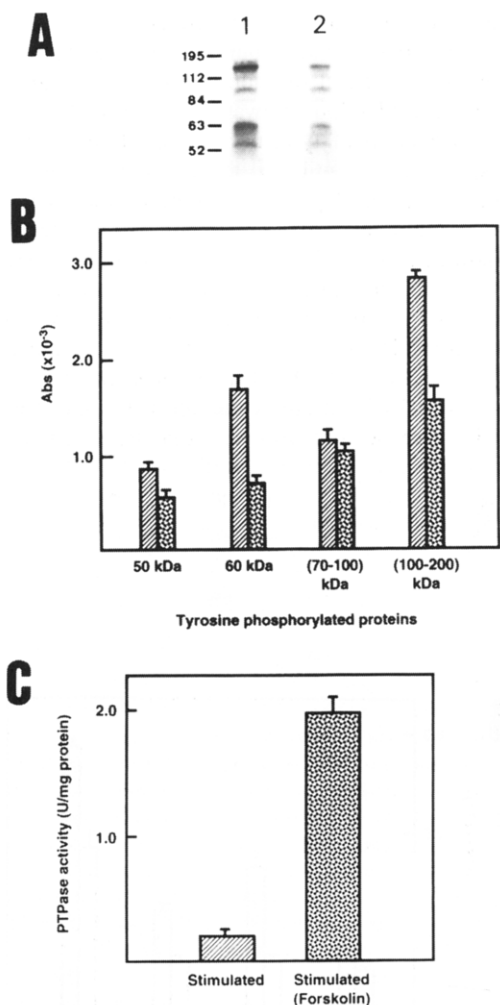
Fig. 2. Effect of forskolin on endothelin induction of tyrosine phosphorylation and on PTP activity. The stimulation of platelets with endothelin-1 was performed in the absence (lane 1) or presence (lane 2) of 10 μM forskolin, as described under Section 2. A: Tyrosine phosphorylated platelet proteins were analyzed on 12% SDS-PAGE and the Western blotting was performed with PY20 antibody. B: Tyrosine phosphorylation level in the different molecular mass ranges of proteins in endothelin-stimulated platelets in the presence (dotted (right) columns) and absence of forskolin (shaded (left) columns). C: PTP activity of endothelin-stimulated platelets in the presence (dotted (right) column) (2.05 U/mg protein) and absence (shaded (left) column) (0.18 U/mg protein) of forskolin. The enzymatic activity was measured as described under Section 2, using 20 μg of platelet proteins at 37°C for 25 min. Results are representative as mean ± SE of four separate experiments.

same samples (Fig. 1C), the increase in tyrosine phosphorylation induced by endothelin-1 was correlated with a significant inhibition of this activity (60%). The different level of tyrosine phosphorylation found here may be explained by a possible endothelin-induced regulatory effect of protein phosphatases that results in a different phosphorylation/dephosphorylation rate due to the coordinated action of protein tyrosine kinases and phosphatases. Taking into account that activation of platelets by several agonists results in tyrosine phosphorylation for several cellular proteins including the FAK [29], we investigated whether the group of proteins in the molecular mass range of 100–200 kDa includes FAK protein. Results shown in Fig. 1A demonstrate that tyrosine phosphorylation of this protein is linked to endothelin-1 effect in rabbit platelets.

As previously proposed [22], endothelin-1-stimulated tyrosine phosphorylation in rabbit platelets can be significantly inhibited by agents that raise cAMP levels and, sensibly less by agents that raise cGMP levels, so the involvement of cyclic nucleotides on platelet tyrosine phosphorylation/dephosphorylation rate disbalance could be considered. This possible effect of cyclic nucleotides on this tyrosine phosphorylation stimulation and on PTP activity inhibition by endothelin was studied by performing endothelin stimulation in the presence of forskolin which increases the cAMP level. At a concentration of 10 μM, forskolin produces a marked inhibition of tyrosine phosphorylation induced by endothelin-1 as shown on the Western blot analysis (Fig. 2A). An inhibitory effect was observed for 60 kDa and 100–200 kDa proteins (58% and 47%, respectively) (Fig. 2B) and a slight one for 50 kDa and 70–100 kDa (36% and 10%, respectively). Moreover, the specific activity of the PTP in whole rabbit platelets treated with endothelin-1 in the presence of forskolin was 11.6-fold higher than in untreated platelets (Fig. 2C). In this sense, our results show that the decrease in protein tyrosine phosphorylation in endothelin-1-stimulated-platelets in the presence of forskolin is correlated to the dramatic increase in the PTP activity with this agent. In this regard, changes in cyclic nucleotide levels could affect the coordinated action of protein kinase and phosphatase activities in the signal transduction cascade. In fact, Cirri et al. [27] described that increase of intracellular concentration of cyclic nucleotides could stimulate some tyrosine phosphatase isoenzyme activities in vivo. Moreover, it has been recently described that modulation of cGMP metabolism is a mechanism through which the tyrosine kinase pathway can interact with other signal transduction pathways [30]. Thus we can consider the possibility of an activation or inactivation of more than one cyclic monophosphate nucleotide-dependent protein tyrosine phosphatase following endothelin-1 stimulation as a function of the different responses to tyrosine phosphorylation inhibition of platelet proteins in the presence of agents which modify the levels of these nucleotides.

Considering the possibility of a modulator effect of endothelin-1 on platelet PTP activity, we have studied the effect of this neuropeptide on the PTP-1C that could participate, through its SH2 domains, in signal transduction pathways. Fig. 3A shows the SDS-PAGE analysis of proteins from platelet lysates and from immunoprecipitates with anti-PTP-1C from endothelin-stimulated and resting platelets, subjected to immunoblotting with antibodies to phosphotyrosine (PY20) and to PTP-1C (α-PTP-1C). PTP-1C from platelet lysates and immunoprecipitates, that undergoes a tyrosine phosphorylation process, was subjected to PTP activity assay from endothelin-stimulated and resting platelets (Fig. 3B). These results show a relationship between the increase in the phosphorylation state of PTP-1C and the decrease in phosphatase activity.

PTP-1C is known to be phosphorylated at tyrosine and serine residues [31,32]. It has been suggested that phosphorylation of PTP-1C could play an important role in the regulation of this enzyme [31]. The results shown in the present paper are in agreement with those obtained by Garton and Tonks [33] in which phosphorylation of PTP-PEST (cytosolic PTP) led to a decrease in the phosphatase activity. In contrast, it has been described more recently that tyrosine phosphorylation of PTP-1D is correlated with an enhancement of
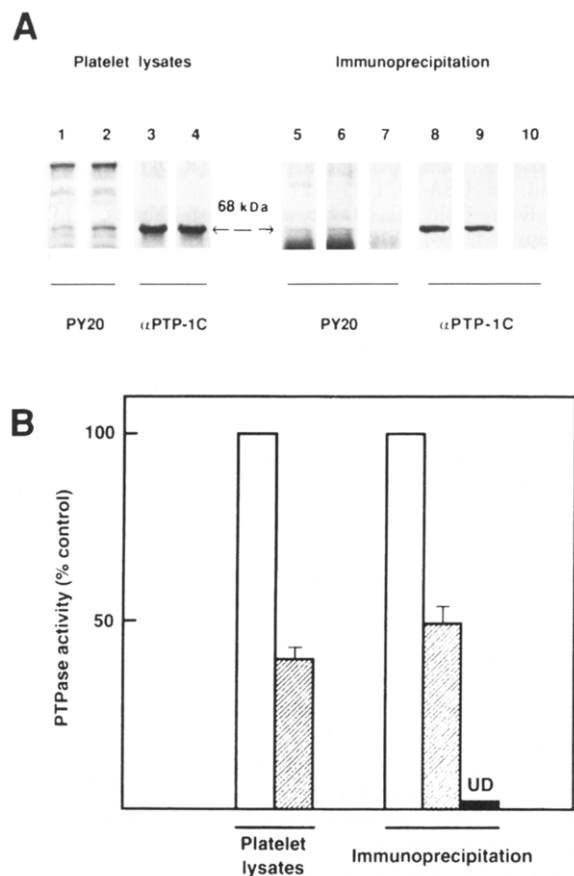
Fig. 3. Analysis of immunoprecipitated PTP-1C from endothelin-stimulated platelets. Platelets were stimulated with endothelin-1 for 30 s as described under Section 2. Immunoprecipitations were performed with anti-PTP-1C (αPTP-1C) and non-immune serum using Protein A-Sepharose. A. Immunoblotting of proteins from platelet lysates and from immunoprecipitates probed with PY20 and αPTP-1C antibodies. Lanes 1, 3, 5 and 8 unstimulated platelets; lanes 2, 4, 6 and 9 endothelin-stimulated platelets. Lanes 7 and 10 correspond to immunoprecipitations with control non-immune serum. Analysis of quantity and coincidence of PTP-1C (lanes 3, 4 and 8 to 10) was performed by autoradiography of the same immunoblot used for PY20 after stripping and reprobing protocols with αPTP-1C. The data shown belong to a single immunoblot representative of three separate experiments of immunoprecipitation. B: Determination of immunoprecipitated PTP-1C activity from resting (□) (0.2 U/mg protein) and endothelin-stimulated platelets (▨) (0.08 U/mg protein). The conditions and the assay method were as described under Section 2 and the activity was measured at 37°C for 25 min. Data represent mean ± SE from three separate experiments. (■) Undetectable PTP activity in control non-immune serum.

its catalytic activity [34]. This discrepancy can be based on the fact that, despite their close structural similarity, the two PTPs have distinct regulatory functions that may be related to their different activity on autophosphorylated receptors or to their ability to form complexes with receptor tyrosine kinases [34].

In any case, the stimulation or inactivation of tyrosine phosphatase activity by a phosphorylation process is one of the signal transduction pathways promoted by G-protein-coupled receptors [35]. This could be correlated to the fact that endothelin receptors belong to this G-protein-coupled class [36] and, in consequence, an interconection between endothelin-1 effect on tyrosine phosphorylation and regulation of PTP activities can be proposed. Moreover, the effect of endothelin-1 on tyrosine phosphorylation of FAK can be con-

sidered an integrin-mediated event in rabbit platelets, as it has been previously described for other agonists [29].

Another feasible possibility is that the endothelin-1 effect on tyrosine phosphorylation and on PTP activity in rabbit platelets could be related with the general idea of a regulation of signal transduction cascades through protein–protein interactions [37] activated by protein tyrosine kinases. In this sense, PTPs contain specific domains (Src homology 2 and 3 or SH2 and SH3) [38] which could be involved in the interactions between cytoplasmic signaling molecules and specific phosphotyrosyl residues on signaling molecules which could provide signal specificity, amplification processes or feed-back mechanisms [39]. PTP-1C particularly contains SH2 domains that allow a possible and direct linkage of this PTP to growth factor receptors, to other signaling proteins or to certain structural cell components, that could end up in the regulation of signal transduction through phosphorylation and dephosphorylation of tyrosine residues [40]. It is believed that intracellular localization is a factor that could control the mechanisms of action of the enzyme. In this regard, it is noticeable to point out that thrombin translocates PTP-1C in platelets [19]. In addition phorbol esters induce translocation and phosphorylation of PTP-1C in HL-60 cells [31]. Thus, we can speculate that endothelin-1 could induce intracellular translocation of PTP-1C in platelets.

Since platelet PTP is extremely sensitive to inhibition by sodium orthovanadate [26], this effect on endothelin-stimulated and resting platelets was studied here. The in vitro results of PTP activity in the presence of different concentrations of orthovanadate with respect to control in the absence of this inhibitor, are shown in Fig. 4. In the presence of 5×10^{-4} M sodium orthovanadate, PTP activity from endothelin-stimulated platelets was inhibited approximately 43%, whereas PTP activity from resting platelets was more sensitive to this concentration of inhibitor (70%), with an IC_{50} of 50 μM and 1 μM, respectively. Thus, our data of a lower catalytic activity of the immunoprecipitated PTP compared to total PTP activity (150-fold, results in mU/mg protein not shown) and a lower sensitivity of PTP for orthovanadate in endothelin-1-stimulated platelets with respect to
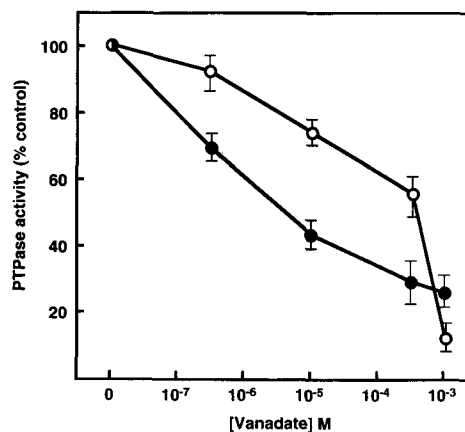

Fig. 4. Dose-response to sodium orthovanadate, a specific inhibitor of PTP activity. PTP activity was measured as described under Section 2 using 20 μg freshly thawed platelet proteins at 37°C for 25 min, in the absence and presence of this inhibitor at the different concentrations indicated, in endothelin-stimulated platelets (○) or in resting platelets (●). Results represent the mean ± SE of three separate experiments.

control make us think of a possible inaccessibility of a conserved cysteine residue that is essential for the PTP activity [41], or of an inhibitory site that implies different sensitivity to vanadate in the same way as it occurs in other cell systems [25] with a different particulate state of PTPs.

In any case, the possible covalent states of platelet PTPs and their different sensitivity to inhibitors suggest that platelets may contain multiple forms of these proteins, and that the presence of an agonist may modify these aspects. Thus, endothelin-1 could affect the possible coordinated action of protein tyrosine phosphatases and kinases in rabbit platelets and modulate, by tyrosine phosphorylation stimulation, some relevant proteins, like focal adhesion kinase through integrin-mediated events and a cAMP dependent pathway.

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