Erbstatin blocks platelet activating factor-induced protein-tyrosine phosphorylation, polyphosphoinositide hydrolysis, protein kinase C activation, serotonin secretion and aggregation of rabbit platelets

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The role of protein-tyrosine phosphorylation in the signal transduction of platelet activating factor (PAF) was investigated in rabbit platelets with a range of synthetic compounds that inhibit protein-tyrosine kinases. In particular, erbstatin ($IC_{50} \sim 20$ μg/ml) abrogated a wide range of platelet responses to PAF, including tyrosine phosphorylation of cellular proteins, polyphosphoinositide turnover, activation of membranous protein kinase C, platelet aggregation, and serotonin secretion. With about a third of the potency of erbstatin, compound RG50864 also inhibited many of these responses, whereas at 100 μg/ml, genistein, 670C88 and ST271 were without effect. Finally, the ability of thrombin to cause platelet aggregation and serotonin secretion was also compromised by erbstatin.

Erbstatin; Protein phosphorylation; Phosphatidylinositol turnover; (Platelet)

1. INTRODUCTION

Like many other cytokines [1], platelet activating factor (PAF) triggers the rapid phospholipase C-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol and inositol-trisphosphate [2–5]. The ability of PAF to transiently increase the cytosolic free Ca^{2+} level in platelets may reflect its mobilization by inositol-trisphosphate [6]. The elevation of Ca^{2+} and diacylglycerol has been linked with the activation of a family of Ca^{2+}-activated, phospholipid-dependent protein kinase (PKC) isozymes [7]. Treatment of rabbit platelets with PAF leads to 2–3-fold increases in both the activities of cytosolic and membrane-bound forms of PKC within one minute [5,8]. The pivotal role that this kinase plays in platelet activation by PAF and other cytokines is implied by the ability of a range of PKC inhibitors to block such downstream responses to these agonists as cell aggregation and the release of mediators like serotonin [9–12].

Within seconds of exposure to human platelets to thrombin and collagen, enhanced tyrosine phosphorylation of several proteins can be detected [13,14]. This implies that the activation of a protein-tyrosine kinase, possibly the receptors for thrombin and collagen or an associated kinase like $pp60^{c-src}$ [15], may participate in early signal transduction leading to platelet activation. The discovery by Rhee and his colleagues [16–18] that a PIP_2-recognizing phospholipase C is tyrosine phosphorylated in cultured cells in response to platelet-derived growth factor and epidermal growth factor, as well as in vitro by the receptor/kinases for these mitogens has raised the possibility that PIP_2 breakdown is directly regulated by protein-tyrosine phosphorylation. While there is a good correlation between the ability of growth factors to induce both phospholipase C phosphorylation and PIP_2 hydrolysis, it is unknown whether the tyrosine phosphorylation is required for activation of the phospholipase.

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Abbreviations: NP40, Nonidet P-40; PAF, platelet activating factor or 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

2. MATERIALS AND METHODS

2.1. *Materials*

Genistein [19] was purchased from Apin Chemicals. Erbstatin [20], 2,5-bis-(3,4-dimethoxybenzylidene)cyclopentanone [21] (670C88), α-cyano-4-hydroxy-3,5-diisopropylcinnamamide [22] (ST271), and α-cyano-3,4-dihydroxythiocinnamamide [23] (RG50864 also numbered 12) were synthesized as published. All of these compounds were prepared as concentrated stock solutions in DMSO, except erbstatin, which was dissolved in ethanol. PAF, thrombin, histone H1 (type III-S), protamine sulphate and most other reagents were bought from Sigma (St. Louis, MO). [2-^{3}H]Inositol and [^{3}H]serotonin were purchased from Amersham (Arlington Heights, IL), and [γ-^{32}P]ATP from ICN Radiochemicals.

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2.2. *Methods*

Rabbit platelets were isolated by the method of Pinckard et al. [24]. Platelets at a concentration of 1×10^8/ml were challenged with PAF (100 pM), or thrombin (2 units/ml) at 37°C in Tyrodes buffer, pH 7.2. Following drug treatment, the 1% NP40-solubilized microsomes from platelets were subjected to MonoQ chromatography, and the column extracts were assayed for protein kinase C activity [8].

The prelabelling of rabbit platelets with [^{3}H]inositol for the polyphosphoinositide turnover studies was performed as indicated [5]. Cells (1.5×10^9/0.5 ml) were challenged with the protein-tyrosine kinase inhibitors for 5 min, then with 100 pM PAF for 1 min. After 1.8 ml of denaturing buffer were added, the various inositol phosphates were extracted and resolved by Dowex-1 (Bio-Rad) chromatography prior to quantitation of radioactivity as described by Watson et al. [25].

For the platelet aggregation studies, cells (2×10^8/ml) were prepared in Tyrode's buffer, pH 7.2 containing calcium chloride (1.3 mM). Aliquots (0.5 ml) were assayed for PAF-induced aggregation in the absence or presence of the protein-tyrosine kinase inhibitors using a Bio-Data aggregometer (Bio-Data, Hatboro, PA). These agonists and inhibitors in 50 μl of 0.25% BSA in Tyrode's buffer were directly added to the platelets in a cuvet. Activities were measured as percent increase in light transmission.

For the serotonin release studies, platelets (2×10^9/ml) were incubated with 0.3 μCi/ml of [^{3}H]serotonin for 3 h at 37°C. The non-incorporated [^{3}H]serotonin was removed by washing the platelets three times with Tyrode's buffer. The platelets (2×10^8/ml) were preincubated in the absence and presence of the protein-tyrosine kinase inhibitors for 5 min and then challenged with 100 pM PAF for 1 min before being pelleted in a microcentrifuge at 15000 rpm for 15 s. The radioactivity remaining in the supernatant was quantitated and plotted against the initial radioactivity present in the cells.

Following sodium-dodecyl-sulfate gel electrophoresis [26], platelet proteins were transferred to a nitrocellulose membrane [27]. After overnight incubation in buffer containing 5% bovine serum albumin and 1% ovalbumin, the blots were incubated with a monoclonal antiphosphotyrosine antibody, PY-20 (ICN), followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Calbiochem). Color development was done using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium (Bethesda Research Laboratories), following the manufacturer's directions.

3. RESULTS

Rabbit platelets are exquisitely responsive to activation by picomolar concentrations of PAF, and within a minute, an optimal dose of PAF (i.e. 100 pM) can trigger the release of more than 80% of total cellular serotonin (Fig. 1). This facile and highly sensitive assay was exploited to test the effects of potential protein-tyrosine kinase inhibitors on PAF responses in rabbit platelets. As shown in Fig. 1, preincubation of platelets for 5 min with erbstatin at concentrations exceeding 10 μg/ml inhibited subsequent PAF-induced serotonin release from the cells. Furthermore, thrombin-elicited serotonin secretion was similarly abolished by erbstatin (not shown). The inhibitor RG50864 also prevented PAF-induced release of serotonin with a third of the potency of erbstatin, whereas genistein, 670C88 and ST271 at 100 μg/ml were without effect on secretion (not shown). All of these compounds have been reported to have negligible effects on protein-serine/threonine phosphorylation [19,22,23,28], while some exhibit selectivity towards different protein-

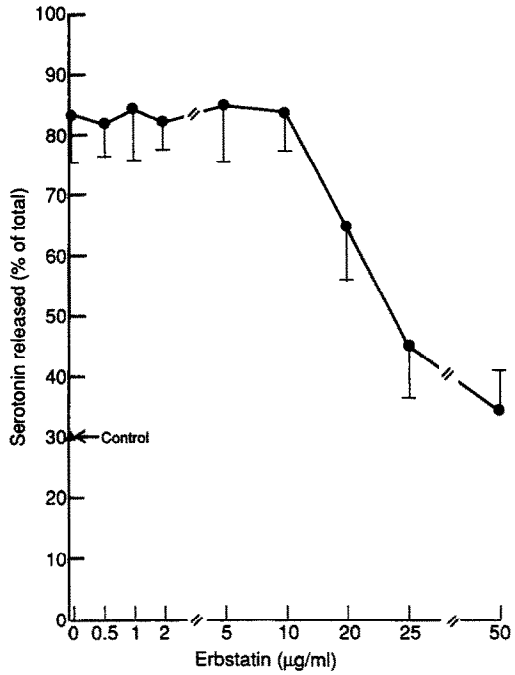

Fig. 1. Dose–response for erbstatin inhibition of serotonin release from PAF-treated rabbit platelets. Cells prelabelled with [^{3}H]serotonin were either untreated (▲) or incubated for 5 min with 0–50 μg/ml erbstatin, and subsequently exposed for 1 min with 100 pM PAF (●) prior to determination of released radioactivity. Values are the means ± SD (n = 8).

tyrosine kinases [22,23]. Since erbstatin produced the most sensitive inhibition of this action of PAF, our latter experiments focused on the effect of this compound on other platelet responses to PAF challenge.

Treatment of platelets with 100 pM PAF induces 50% aggregation within 1 min (Fig. 2). Erbstatin at 25 μg/ml had no effect on cell aggregation by itself, but it abolished the platelet clumping caused by 100 pM PAF (Fig. 2) and 2 units/ml thrombin (not shown).

Protein kinase C has been implicated in the control of platelet aggregation and secretion. Within 1 min of either PAF or thrombin exposure to rabbit platelets, both the cytosolic and 1% NP40-solubilized particulate PKC activities become elevated 2–3-fold as measured in MonoQ-fractionated extracts [5,8]. Regardless of whether the Ca^{2+}, diacylglycerol and phosphatidyl-

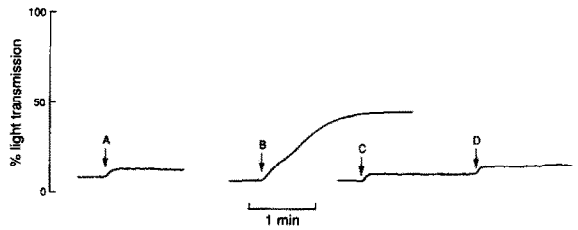

Fig. 2. Erbstatin inhibition of PAF-induced platelet aggregation. Platelet aggregation was measured as described in section 2. (A) Only buffer was added; (B) 100 pM PAF was added; (C) 25 μg/ml erbstatin was added, and then 2 min later (D) 100 pM PAF was added. Results are representative of five independent experiments.

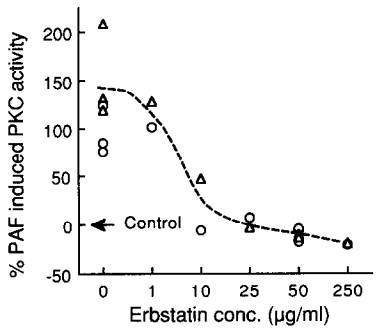

Fig. 3. Erbstatin inhibition of PAF stimulation of particulate-derived protein kinase C activity. Washed platelets were preincubated for 5 min with 0–50 μg/ml erbstatin, and subsequently treated for an additional min with 100 pM PAF prior to harvesting. MonoQ chromatography of 0.5 mg of 1% NP40-solubilized particulate protein from the platelet extracts was performed, and the activity of PKC in the peak fractions (0.26–0.34 M NaCl) was measured towards histone H1 in the presence of Ca^{2+}, diolein and phosphatidylserine (△), and protamine sulfate in the absence of these cofactors (○). Less than 5% of the histone H1 phosphorylating activity was detected in the absence of Ca^{2+}, diolein and phosphatidylserine (not shown).

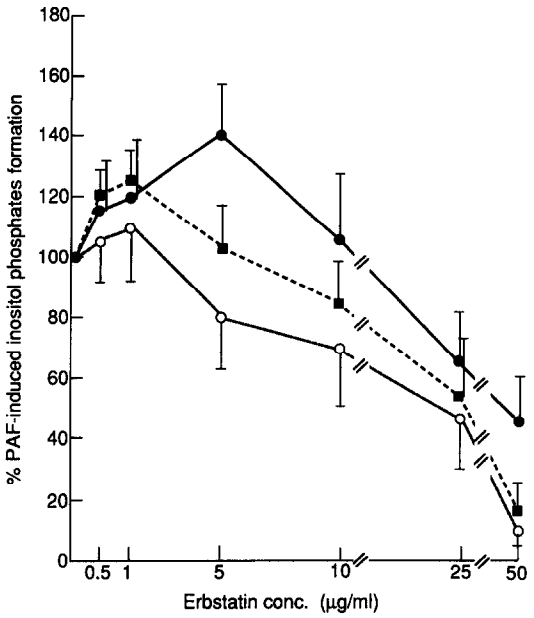

Fig. 4. Erbstatin inhibition of PAF-induced polyphosphoinositide breakdown. Rabbit platelets that were prelabelled with [^{3}H]inositol were subsequently incubated for 5 min in the presence of 0–50 μg/ml erbstatin, and subsequently treated for 1 min with 100 pM PAF prior to harvesting. The percent increases of PAF-induced incorporation of radioactivity into inositol mono- (●), bis- (■) and trisphosphate (○) are shown. Values are the means ± SD of at least 10 determinations per point.

serine-dependent histone H1 phosphorylating or cofactor-independent protamine phosphorylating activities of PKC were assessed, the ability of PAF to stimulate the particulate-derived PKC activity was blocked by erbstatin concentrations exceeding 10 μg/ml (Fig. 3). Similarly, the enhanced cytosolic PKC activity from PAF-treated platelets was also lost if the cells were pretreated with erbstatin (not shown).

The erbstatin-mediated inhibition of PKC activation by PAF indicated that this compound might also interfere with the ability of PAF to induce PIP_2 breakdown, since this has been implicated as the preceding step in the signal transduction pathway leading to PKC. As shown in Fig. 4, at 5 μg/ml and higher concentrations of erbstatin, PAF-induced formation of inositol mono-, bis- and trisphosphates was progressively inhibited.

As thrombin and collagen had been shown to stimulate protein-tyrosine phosphorylation in human platelets [13,14], and the erbstatin experiments implied that this form of covalent modification might mediate PAF action, the ability of PAF to stimulate the tyrosine phosphorylation of rabbit platelet proteins was examined. PAF treatment of the platelets led to the rapid phosphorylation of several polypeptides as assessed by Western blotting with anti-phosphotyrosine antibodies (Fig. 5). Although not shown in this figure, a parallel incubation with antiphosphotyrosine antibodies in the presence of 2 mM phosphotyrosine completely eliminated the labelling, whereas 2 mM phosphoserine only slightly decreased this signal. Phosphoamino acid analysis performed on at least 8 phosphoproteins whose phosphorylations were increased in response to PAF in ^{32}P-labelled cells confirmed that they were tyrosine phosphorylated (not shown). The major polypeptides whose phosphorylation was stimulated by PAF and inhibited by erbstatin preincubation, are indicated in Fig. 5. The dose–response of erbstatin inhibition of several polypeptides generally correlated with its inhibitory action on the other platelet responses to PAF.

4. DISCUSSION

Erbstatin was originally identified in the medium of an actinomycete as a compound that inhibited the autophosphorylation of the EGF receptor in membranes of A431 epidermoid carcinoma cells with an $IC_{50} < 2.5$ μg/ml [29]. Unlike genistein, which acts as a competitive inhibitor with respect to ATP [19], erbstatin may compete instead with the phosphoacceptor protein to reduce its phosphorylation by the EGF receptor/protein-tyrosine kinase [28]. The protein-tyrosine kinase inhibitor erbstatin was found to inhibit all of the PAF-stimulated responses measured in rabbit platelets with an IC_{50} of ~20 μg/ml. This is about 30 times the concentration reported for 50% inhibition of the EGF receptor/protein-tyrosine kinase [28]. On the other hand, a wide range of erbstatin analogs [23] have been shown to inhibit the insulin receptor/protein-tyrosine kinase with 100–700-fold less potency than the EGF receptor. It may be that another erbstatin analog, not tested in this study, could serve to better inhibit

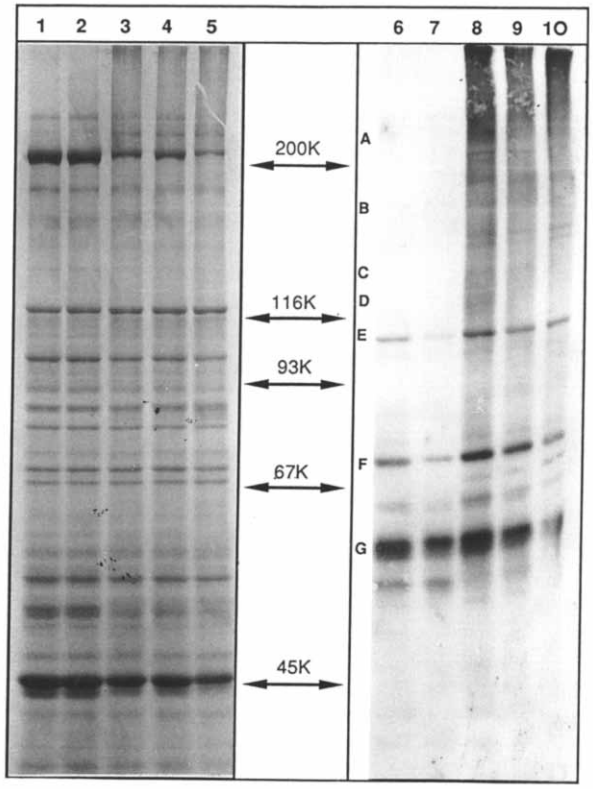

Fig. 5. Immunoblot with antiphosphotyrosine antibodies of phosphoproteins from PAF-treated platelets incubated with 10 or 25 μg/ml erbstatin. Washed platelets were untreated (lanes 1 and 6), or treated with 10 μg/ml erbstatin (lanes 2 and 7), 50 pM PAF for 1 min (lanes 3 and 8), 10 μg/ml erbstatin for 5 min followed by 50 pM PAF (lanes 4 and 9), or 25 μg/ml erbstatin for 5 min followed by 50 pM PAF for 1 min (lanes 5 and 10). Cells were rapidly centrifuged and the pellets frozen. Pellets were solubilized in SDS sample buffer and the equivalent of 8×10^6 cells were loaded per lane on a 7.5% polyacrylamide gel and electrophoresed. Lanes 1–5 are from a Coomassie blue-stained gel and lanes 6–10 are from a Western blot probed with antiphosphotyrosine antibodies. The major polypeptides whose labelling were increased in response to PAF and at least partially inhibited by erbstatin are indicated by letters. Their M_rs are as follows: A, 215000; B, 172000; C, 137000; D, 123000; E, 108000; F, 70000; G, 56000.

pp60^{src} or whatever kinase is stimulated in response to PAF in platelets. RG50864, which inhibited the PAF responses in platelets with about 3-fold less potency than erbstatin, has been reported to inhibit the EGF receptor/protein-tyrosine kinase about three times more strongly than erbstatin [23].

Our findings indicate that increased protein-tyrosine phosphorylation is an important component in the post-receptor signal transduction system for PAF, and quite possibly thrombin. While treatment of platelets with erbstatin was not able to completely inhibit the increased tyrosine phosphorylation in response to PAF, it was clear that phosphorylation of a number of polypeptides was diminished by this inhibitor. Since the receptors for PAF and thrombin have not yet been isolated, it remains feasible that they feature protein-tyrosine kinase catalytic domains or alternatively they may associate with protein-tyrosine kinases. Akin to the EGF and PDGF receptors [16–18], the tyrosine phosphorylation of an associated phospholipase C might facilitate the hydrolysis of PIP_2 and activation of PKC.

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