Alkaline phosphatase prevents platelet stimulation by thromboxane-mimetics

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1 The effects of alkaline phosphatase on platelet aggregation, secretion and thromboxane B₂ (TxB₂) generation induced by the full dose-range of common platelet agonists were studied in human platelet-rich plasma and washed platelets.

2 Platelet aggregation and adenosine 5'-triphosphate (ATP) secretion induced by threshold and supra-maximal concentrations of arachidonate and stable TxA₂ and prostaglandin endoperoxide-mimetics (compounds U46619 and EP171) were abolished in the presence of alkaline phosphatase (0.5–1 u ml⁻¹), even though the synthesis of TxB₂ persisted. In contrast, platelet aggregation by PAF-acether and by supramaximal concentrations of thrombin as well as the primary wave of aggregation by adenosine diphosphate (ADP) and adrenaline were unaffected by alkaline phosphatase under conditions where the secondary wave of aggregation by ADP was blocked.

3 Alkaline phosphatase, unlike prostacyclin, failed to raise the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of the platelets. Also, the pretreatment of platelets by inorganic phosphate or by ATP plus creatine phosphate/creatine phosphokinase reversed the inhibitory effect of alkaline phosphatase.

4 Experiments performed in the guinea-pig *in vivo* showed that alkaline phosphatase was effective on thrombocytopenia induced by arachidonate.

5 Our results provide the first direct evidence for a specific inhibitory effect of alkaline phosphatase at a site sensitive to TxA₂ and prostaglandin endoperoxides and suggest that its phosphorylation/dephosphorylation state may play an important role in modulating platelet activation. These results also suggest the presence of ecto-protein kinases on membrane platelets.

Keywords: Alkaline phosphatase; platelet activation; arachidonate; thromboxane A₂; prostaglandin endoperoxide-mimetics; ecto-protein kinases

Introduction

Platelets change shape, aggregate and release their granule contents in response to a wide variety of activating agents (Holmsen, 1965; Caen & Michel, 1972; Kinlough-Rathbone *et al.*, 1977), but the mechanisms involved in these reactions are not completely understood. Platelet activation, by many external stimuli, is associated with phosphorylation and dephosphorylation reactions (Lyons *et al.*, 1975; Haslam & Lynham, 1977; Haslam *et al.*, 1979; Daniel *et al.*, 1981; Imaoka *et al.*, 1983; Schafer *et al.*, 1986; Touqui *et al.*, 1986). This has led us to study the possibility that the dephosphorylating enzyme alkaline phosphatase, by removing phosphate groups from the external platelet membrane, might modulate the platelet function.

In this paper, alkaline phosphatase was used to induce the dephosphorylation of the external surface of the platelets which may be related to its activation. Our data show that alkaline phosphatase prevents platelet aggregation and secretion by thromboxane-mimetics and suggest the dephosphorylation of ecto-proteins.

Methods

Preparation of human platelets

Blood obtained from healthy human volunteers, who had not received any medication for at least 10 days, was mixed with citrate (0.38%, final concentration) to prevent coagulation. Platelet-rich plasma (PRP) was obtained by standard differen-

tial centrifugation (200 *g*, 20 min). In order to wash platelets, blood was anticoagulated with 1/6th of its final volume of citric acid-citrate-dextrose (7, 93 and 139 mM, respectively, pH 6.4) containing heparin (20 u ml⁻¹). PRP prepared as above was centrifuged (1700 *g*, 15 min, 37°C) and the platelet pellet was resuspended and washed twice with a modified Tyrode buffer containing bovine serum albumin (0.35%), according to Mustard *et al.* (1972), in which apyrase was omitted and prostacyclin (PGI₂, 1 nM) was added for the first two steps of washing. The platelets were resuspended in the same buffer, their number was determined with a Coulter Counter ZBI and adjusted to a final concentration of 5 × 10⁸ cells ml⁻¹.

Platelet stimulation

Platelet aggregation was measured turbidimetrically (Born, 1962) at 37°C in an Icare aggregometer (Marseille, France) on 0.4 ml samples of PRP or washed platelets, stirred at 1100 r.p.m. Platelets were challenged with different agonists alone or in the presence of alkaline phosphatase. When washed platelets were used (except for thrombin), fibrinogen was added at a final concentration of 0.28 mg ml⁻¹. ATP secretion was determined at 3 min, after adding the stimulating agent, by the bioluminescence technique (luciferine-luciferase) (Holmsen *et al.*, 1972) using a Pico-ATP device from Jobin Yvon (Paris, France). At 5 min, when arachidonate was used, 100 µl samples were collected for the further radioimmunoassay of thromboxane B₂ (TxB₂) bioformation.

Radioimmunoassay of thromboxane B₂

TxB₂ was determined according to Sors *et al.* (1978). Briefly, platelet samples containing TxB₂ were diluted in radioimmunoassay phosphate-gelatin buffer, together with [¹²⁵I]-TxB₂

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and TxB₂-antiserum. After overnight incubation at 4°C, bound fractions were separated by precipitation with polyethylene glycol 6000 and after centrifugation the pellet radioactivity was counted.

Measurement of adenosine 3':5'-cyclic monophosphate (cyclic AMP)

The samples were treated with perchloric acid 1 N and, following appropriate dilution in 0.1 M sodium citrate buffer at pH 6.2, acetylated and assayed for cyclic AMP by radioimmunoassay as described by Cailla *et al.* (1973). The total amount of cyclic AMP was expressed in pmol mg⁻¹ of protein measured by method of Lowry *et al.* (1951).

Evaluation of thrombocytopenia and bronchoconstriction in guinea-pigs in vivo

Thrombocytopenia and bronchoconstriction in anaesthetized guinea-pigs were studied as described by Lefort & Vargaftig (1978). 5-Hydroxytryptamine (5-HT, 3 µg/animal) was used as a standard bronchoconstrictor agent. Arachidonate (750 µg kg⁻¹) or PAF (33 ng kg⁻¹) was given i.v. 60 min after the injection of alkaline phosphatase (AP, 7 u ml⁻¹) or its solvent (Tris). When arachidonate was studied, aspirin (acetylsalicylic acid, 20 mg kg⁻¹) was used instead of alkaline phosphatase. The bronchopulmonary resistance to inflation (bronchoconstriction) following the administration of the agonists was expressed in cmH₂O, as its peak value.

Statistical analysis

Statistical significance was analysed by Student's *t* test for unpaired data. *P* values of less than 0.05 were considered to be significant. Results are expressed as means ± s.e.mean.

Materials

The stable endoperoxide/thromboxane A₂ (TxA₂) analogues U46619 and EP171 were a gift from Upjohn Co (Kalamazoo, U.S.A.) and from Prof. R.L. Jones, Department of Pharmacology (University of Edinburgh Medical School, Great Britain), respectively. Arachidonic acid (AA), adenosine diphosphate (ADP), adrenaline, prostacyclin (PGI₂), cyclic AMP, kidney bovine alkaline phosphatase, bovine serum albumin (Fraction V), bovine thrombin, creatine phosphate (CP) and creatine phosphokinase (CPK) were purchased from Sigma. Heparin was from Laboratories Choay (Paris, France). PAF (1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine) was from Bachem (Bubendorf, Switzerland) and collagen from Hormon Chemie (Münich, Federal Republic of Germany). Fibrinogen (Grade L) from Kabi (Stockholm, Sweden) was treated with diisopropyl fluorophosphate (DFP) by standard procedures to inactivate coagulant contaminants. Stock solutions of arachidonate, U46619 and EP171 in ethanol were stored at -20°C, dilutions being freshly prepared before each experiment. PGI₂ was dissolved in 0.05 M Tris buffer, pH 8.5. Thrombin was solubilized in Michaelis Owren-Koller buffer (30 mM sodium veronal, 44 mM sodium acetate, 108 mM NaCl; pH was adjusted to 7.3 with HCl 0.1 N).

Results

When plasma-free washed human platelets were incubated with alkaline phosphatase (0.1–1 u ml⁻¹) as indicated in the legend of Figure 1a and b, both ATP secretion and aggregation triggered by the natural (arachidonate-derived) and by the synthetic prostaglandin endoperoxide and thromboxane analogues U46619 (1 µM) and EP171 (0.01 µM) were blocked (Figure 2). As shown in Figure 1a and b, inhibition of aggregation induced by arachidonate and by compound EP171 was concentration-dependent; these figures also show that denatured (heated) alkaline phosphatase loses its ability to

Figure 1 Representative tracings of platelet aggregation by arachidonate (AA) and by the thromboxane and endoperoxide analogues (U46619 and EP171). Inhibition by alkaline phosphatase (AP) of AA (a), EP171 (b) and U46619 (c and d)-induced platelet aggregation. Heating of AP at 100°C for 2 min removed antagonistic activity (denat.), the curves were not different from control. A time course experiment is shown in (d). At a concentration of 0.01 u ml⁻¹, AP failed to block aggregation by U46619 when incubated with the platelets for 2 min, but became effective when incubation was prolonged to 10 min. The concentration of 0.1 u ml⁻¹ of AP was already effective when incubated with the platelets for 2 min, and showed a reduced activity when the duration of incubation was shorter (30 or 15 s). Generation of thromboxane B₂ (TxB₂), the stable degradation product of TxA₂, was assayed as described in Methods. Platelets pre-exposed to 0.5 u ml⁻¹ AP and then stimulated with 0.1 mM AA generated amounts of TxB₂ (439.7 ± 88.2 ng ml⁻¹) not significantly different from control platelets (495.4 ± 95.2), (means ± s.e.mean, *n* = 4).

interfere with platelet function. Inhibition of aggregation by compound U46619 was concentration- and time-dependent when alkaline phosphatase was used at the sub-maximal concentration of 0.1 u ml⁻¹ for 15 s–2 min (Figure 1c and d). Alkaline phosphatase also blocked aggregation by arachidonate, U46619 and EP171 when tested on platelet-rich plasma (PRP; data not shown).

Aggregation of human platelets by ADP and adrenaline occurs in the form of a double wave. The secondary wave of aggregation and the accompanying release reaction are inhibited by aspirin-like drugs (O'Brien, 1968; Mills *et al.*, 1974) and accordingly, they are attributed to the formation of pro-aggregatory metabolites of arachidonate, TxA₂ and prostaglandin endoperoxides (Holmsen, 1977; Lages & Weiss, 1980; and Meyers *et al.*, 1979). The primary wave of aggregation by ADP persisted in the presence of alkaline phosphatase, but the secondary wave and the accompanying ATP secretion were abolished. Thus, in three experiments, maximal aggregation by 10 µM ADP was 78% of maximal in the absence, and only 30% of maximal in the presence of alkaline phosphatase. In contrast, aggregation triggered by adrenaline (0.1 µM) and by the phospholipid PAF (1 µM) was not modified by alkaline phosphatase (Figure 3). Effectiveness of alkaline phosphatase against arachidonate as well as its stable mimetics U46619 and EP171 suggested that alkaline phosphatase acts

Figure 2 Aggregation and release of ATP after platelet stimulation by arachidonate (AA, $100\ \mu\text{M}$), U46619 ($1\ \mu\text{M}$), EP171 ($0.01\ \mu\text{M}$) or thrombin ((1) $0.1\ \text{u ml}^{-1}$, (2) $1\ \text{u ml}^{-1}$) in absence (hatched columns) or presence (stippled columns) of alkaline phosphatase (AP, $0.5\text{--}1\ \text{u ml}^{-1}$) for 2 min. Platelet aggregation was determined as in % light transmission across the platelet suspension described in Methods. Release of ATP was monitored with the luciferin-luciferase technique and expressed in nmol ml^{-1} . Results are the mean (s.e.mean shown by vertical bars) of three separate experiments. Significantly different (** $P < 0.01$; *** $P < 0.001$) when compared to control platelets.

as an antagonist rather than as a cyclo-oxygenase inhibitor and indeed, alkaline phosphatase failed to interfere with the formation of TxA_2 from arachidonate (see legend of Figure 1).

Platelets exposed to alkaline phosphatase and then washed and resuspended behaved as controls, demonstrating that its effects are reversible. Platelet aggregation and ATP secretion induced by low concentrations of thrombin ($0.1\ \text{u ml}^{-1}$) were inhibited by their preexposure to alkaline phosphatase, whereas responses to high concentrations of thrombin ($1\ \text{u ml}^{-1}$) were unaffected (Figure 2). Similar results were obtained with collagen (data not shown). Finally, as shown in

Figure 3 Representative tracings of platelet aggregation in platelet-rich plasma induced by ADP ($1\ \mu\text{M}$), adrenaline (Ad; $0.1\ \mu\text{M}$) or PAF ($1\ \mu\text{M}$) and inhibition by alkaline phosphatase (AP; $1\ \text{u ml}^{-1}$) added 2 min before the addition of each agonist. In control platelets, aggregation was performed in the presence of alkaline phosphatase solvent (Tris).

Table 1, alkaline phosphatase, unlike prostacyclin, did not raise basal cyclic AMP levels. Exposure of platelets to ATP (a substrate for phosphorylation reaction) and to the enzyme system creatine phosphate (CP)/creatine phosphokinase (CPK) that is used to convert released ADP to ATP (Kinlough-Rathbone *et al.*, 1977) abolished the alkaline phosphatase inhibiting activity on U46619-induced platelet aggregation. Comparable results were obtained when inorganic phosphate, an inhibitor of alkaline phosphatase activity, was used (Figure 4).

In the *in vivo* experiments, thrombocytopenia induced by the administration of arachidonate to the guinea-pigs was suppressed for more than 2 h after the i.v. administration of alkaline phosphatase ($7\ \text{u ml}^{-1}$ of blood; the volume of blood was estimated to $1/10$ th of body weight). In contrast, this enzyme did not interfere with bronchoconstriction ($22 \pm 3\ \text{cmH}_2\text{O}$ for alkaline phosphatase-pretreated animals versus $23 \pm 3\ \text{cmH}_2\text{O}$ for control animals; $n = 3$ for each group) and thrombocytopenia ($8 \pm 4\%$ reduction of counts for pretreated animals versus 48 ± 4 for control animals; $n = 3$ for each group) due to the i.v. administration of arachidonate. As expected, aspirin at $20\ \text{mg kg}^{-1}$, i.v. abolished both bronchoconstriction and thrombocytopenia induced by arachidonate. Alkaline phosphatase also failed to interfere with bronchoconstriction ($25 \pm 2\ \text{cmH}_2\text{O}$ for pretreated animals versus $24 \pm 4\ \text{cmH}_2\text{O}$ for control animals; $n = 3$ for each group) and thrombocytopenia ($74 \pm 6\%$ reduction of counts for pretreated animals versus 81 ± 5 for control animals; $n = 3$ for each group) due to PAF (Figure 5).

Discussion

Alkaline phosphatase behaves as a selective antagonist of platelet activation by the pro-aggregatory metabolites of arachidonate, by its mimetics and releasing agents (low thrombin and collagen). The enzymatic activity of alkaline phosphatase was required for its effectiveness, since denatured enzyme (which loses the ability to dephosphorylate *p*-nitro-

Table 1 Effects of alkaline phosphatase (AP) and prostacyclin (PGI_2) on the formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) by platelets

	Cyclic AMP (pmol mg^{-1} protein)		
	Solvent	AP	PGI_2
Saline	21.4 ± 4.7	27.5 ± 6.7	$388.2 \pm 39.3^{***}$
U46619	23.4 ± 5.8	26.8 ± 7.7	$348.8 \pm 40.6^{***}$

Aliquots of suspended platelets were stirred at 37°C and incubated with PGI_2 ($1\ \mu\text{M}$), AP ($1\ \text{u ml}^{-1}$) or the solvent (Tris) alone or in the presence of U46619 ($1\ \mu\text{M}$). The reaction was stopped after 2 min by perchloric acid $1\ \text{N}$ and the cyclic AMP content was measured as previously described.

The results are the mean $\pm$ s.e.mean of four separate experiments. Significantly different (** $P < 0.01$; *** $P < 0.001$) when compared to solvent-treated platelets (control).

Figure 4 Representative tracings of platelet aggregation by the endoperoxide analogue U46619. Reversal by inorganic phosphate (Na_2HPO_4 , 1 mM) and by ATP (0.01 mM) plus creatine phosphate (CP)/creatine phosphokinase (CPK) (0.7 mM and 14 u ml^{-1} , respectively) of the inhibitory effect of alkaline phosphatase. Washed platelets were incubated with alkaline phosphatase alone and in the presence of inorganic phosphate or ATP plus CP/CPK for 2 min before the addition of U46619 (1 μM).

phenylphosphate) failed to modify platelet responsiveness. In agreement with the ability of alkaline phosphatase to suppress the effects of arachidonate, activation of plasma-free platelets by low concentrations of thrombin (0.1 u ml^{-1}) which is accounted for by cyclo-oxygenase metabolites (Packham *et al.*, 1973; Vargaftig, 1977; Lapetina *et al.*, 1978), was blocked by alkaline phosphatase, whereas activation by higher concentrations, known to be cyclo-oxygenase-independent (Packham

et al., 1973; Vargaftig, 1977; Lapetina *et al.*, 1978), persisted (Figure 3). Comparable results were obtained with collagen, since reduced concentrations (0.5–2 $\mu\text{g ml}^{-1}$), but not higher concentration (5 $\mu\text{g ml}^{-1}$), were also inhibited by alkaline phosphatase (data not shown).

Overall, the antagonism by alkaline phosphatase of platelet activation by some, but not all, stimulating agents and the ineffectiveness of the heated enzyme rules out a non-specific mode of action for alkaline phosphatase, such as a detergent effect. In agreement, alkaline phosphatase also failed to deaggregate platelets, when added 2 min or later after the stimulating agents, under conditions where prostacyclin and bivalent ion chelation with EDTA are effective. Finally, unlike prostacyclin, alkaline phosphatase did not raise basal platelet cyclic AMP levels (Table 1), ruling out the possibility that inhibition involves the second messenger system.

As noticed above, platelets exposed to alkaline phosphatase and then washed and resuspended behaved as controls, demonstrating that its effects are reversible. This finding also argues against the possibility that alkaline phosphatase was contaminated with proteases which could cleave irreversibly either the TxA_2 receptor or other membrane proteins to inhibit platelet aggregation. Reversibility also supports the concept that the sites of interaction of the platelet with the arachidonate metabolites and its mimetics involve the external surface of the cell. This antagonism to the effects of TxA_2 should explain the interference of alkaline phosphatase with the second wave of ADP-induced aggregation, which is suppressed by thromboxane agonists and cyclo-oxygenase inhibitors (O'Brien, 1968; Mills *et al.*, 1974; Armstrong *et al.*, 1985). Those inhibitors are also effective against the second wave of adrenaline-induced aggregation (O'Brien, 1968; Armstrong *et al.*, 1985), which was surprisingly not modified by alkaline phosphatase. This unexpected dissociation between ADP and adrenaline is probably accounted for by different locations of their receptor site(s) within the membrane and/or by divergent transduction mechanisms underlying platelet activation even though in both cases cyclo-oxygenase-dependent metabolites of arachidonate are involved. It is likely that these sites participate with the second wave of aggregation triggered by ADP, since they are alkaline phosphatase-sensitive, whereas equivalent (also cyclo-oxygenase-dependent) sites for adrenaline, were less accessible and are thus probably located more internally within the cell.

The reversibility of the inhibitory effect of alkaline phosphatase as well as its reversion by ATP + CP/CPK or by Na_2HPO_4 , support the hypothesis that strategic phosphate groups were accessible from the outside to protein-kinase and phosphatase activities, respectively. Besides, in the *in vivo* experiments in guinea-pigs, bronchoconstriction following the injection of arachidonate, unlike thrombocytopenia, was not suppressed by alkaline phosphatase. The protective effect of alkaline phosphatase seems specific since bronchoconstriction and thrombocytopenia following the i.v. injection of PAF were not modified by this enzyme. As expected, aspirin which suppresses the formation of bioactive metabolites of arachidonate via cyclo-oxygenase, inhibited altogether bronchoconstriction and thrombocytopenia induced by arachidonate.

Our results indicate that alkaline phosphatase interferes selectively *in vitro* and *in vivo* with platelet activation, probably by modifying the phosphorylation/dephosphorylation state of the recognition site for the prostaglandin endoperoxides/ TxA_2 , but we cannot rule out a direct interaction with this site, by a competitive mechanism. Both possibilities are in fact not in contradiction, since the enzymatic activity requires prior enzyme fixation to the site of action. In addition, our results suggest that ecto-protein kinases exist on platelets.

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Figure 5 Inhibition by alkaline phosphatase (AP) of thrombocytopenia, but not of bronchoconstriction, due to the administration of arachidonate (AA) to the guinea-pig. Typical tracings of bronchoconstriction (upwards pen deflection, in cmH_2O) and thrombocytopenia (in parentheses, % reduction of number of free platelet counts in blood, 1 min after AA or PAF administration) representative of three separate experiments each. For these tracings, five pentobarbitone-anaesthetized guinea-pigs were challenged with 5-hydroxytryptamine (5-HT; i.v. at 3 $\mu\text{g}/\text{animal}$) as a standard bronchoconstrictor agent. AA (750 $\mu\text{g kg}^{-1}$, left panel) or PAF (33 ng kg^{-1} , right panel) were given i.v. 60 min after the injection of AP (7 u ml^{-1}), its solvent (Tris) or aspirin (ASA, 20 mg kg^{-1} , only when AA was used).

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