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ADP receptor antagonists inhibit platelet aggregation induced by the chemokines SDF-1, MDC and TARC

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Abstract The ability of the chemokines SDF-1, MDC and TARC to induce platelet aggregation depends strongly on low levels of ADP. The ADP receptors involved have now been characterized using the $P2Y_1$ and $P2T_{AC}$ receptor antagonists, A2P5P and AR-C69931MX. Stimulation of aggregation by the chemokines at 10 s was not blocked by AR-C69931MX, but was strongly inhibited by A2P5P. Pertussis toxin abolished the chemokine-stimulated aggregation. We conclude that the $P2Y_1$ ADP receptor plays a critical role in the initial phases of SDF-1-, MDC- and TARC-induced platelet aggregation, which involve a pertussis toxin-sensitive G protein.

Key words: Blood platelet; Aggregation; Antagonist; Chemokine; ADP receptor

1. Introduction

Blood platelets play important roles in homeostasis, minimizing blood loss and promoting wound healing [1]. In addition to these essential roles of platelets, they also contribute to the development of atherosclerosis and may be involved in inflammation and infection [1,2]. During such conditions, platelets may be activated, adhering to sites of injury, aggregating to form large fibrin-rich thrombi and releasing granule contents. These granules contain inflammatory mediators such as chemokines as well as highly adhesive proteins such as von Willebrand factor, fibrinogen and P-selectin [3–5].

Chemokines are low-molecular weight proteins with important roles, especially in response to tissue damage or infection [6,7]. They exist in two main families according to the positions of conserved cysteine residues; CXC chemokines such as IL-8 (interleukin-8) and SDF-1 (stromal-derived factor-1), and CC chemokines such as MDC (macrophage-derived chemokine), TARC (thymus and activation-regulated chemokine) and RANTES (regulated on activation, normal T cell expressed and secreted). Chemokines are ligands for G protein-coupled receptors whose activation may mediate the migration of leukocytes to the sites of injury and inflammation [7,8]. Recently, CXCR4, a receptor for SDF-1, and CCR4, a receptor for MDC and TARC, have been shown to be expressed on platelets, and SDF-1, MDC and TARC can initiate platelet aggregation [9–11]. However, this ability is strongly influenced by the presence of low levels of the primary platelet agonist ADP [11]. Low levels of ADP may be present in blood [12], enabling platelet activation and aggregation when chemokines are released into the circulation during infection and inflammation.

ADP is a primary platelet agonist which also enhances the activation induced by other agonists such as thrombin and collagen [13]. Moreover, platelet aggregation induced by ADP can be greatly enhanced by epinephrine (EPI) which by itself cannot induce platelet aggregation [14]. These observations support the hypothesis that ADP is an especially important factor for platelet function. Recently, several ADP receptors on platelets have been described [15–18], including the P_2 receptors $P2Y_1$ and $P2T_{AC}$ which are coupled to the $G_{\alpha q}$ and $G_{\alpha i}$ proteins, respectively, and activation of both receptors appears necessary for ADP-induced platelet aggregation [15]. In the present study, we have investigated which ADP receptors are involved in platelet activation by SDF-1, MDC and TARC. Along with these chemokines, we also examined EPI, which is a well-known coagonist as a model to compare the effects of EPI and the chemokines.

2. Materials and methods

2.1. Materials

SDF-1, MDC and TARC were obtained from R&D Systems (Minneapolis, MN, USA) and from Research Diagnostics, Inc. (Flanders, NJ, USA). MDC was also provided by Drs. D. Chantry and P. Gray of the ICOS Corporation (Bothel, WA, USA). AR-C69931MX was obtained from R+D Charnwood AstraZeneca (Loughorough, Leics, UK). Pertussis toxin (Ptx) A protomer was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA). Apyrase (grade VII), indomethacin, prostacyclin (PGI_2), EPI, A2P5P and other speciality chemicals were from Sigma (St. Louis, MO, USA).

2.2. Preparation of platelets

Human venous blood was collected and anticoagulated with acid-citrate dextrose (ACD) for a final citrate concentration of 11.5 mM [19]. Platelet-rich plasma (PRP), prepared as described by centrifugation at $350\times g$, was mixed with ACD (11.5 mM), apyrase (5 U/ml), indomethacin (1 mg/ml) and PGI_2 (0.3 μg/ml), and centrifuged at $620\times g$ for 20 min. The resulting platelet pellet was washed with ACD (1/2 volume of PRP) containing albumin (3 mg/ml) and apyrase (5 U/ml) and centrifuged at $620\times g$ for 20 min. The final platelet pellet was suspended in an Eagle's/HEPES buffer containing 0.5 mg/ml fibrinogen [20]. The final platelet concentrations were typically 3.2×10^8/ml, with a yield of 80–90% compared to PRP, and were

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Abbreviations: SDF-1, stromal-derived factor-1; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; EPI, epinephrine; Ptx, pertussis toxin

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stored at room temperature. All experiments were completed within 5 h.

2.3. Platelet aggregation

To assess platelet aggregation, 35 μl of washed platelets were preincubated for 10 min with saline, apyrase (5 U/ml) or the ADP antagonists A2P5P or AR-C69931MX at 37°C. The preincubated platelets were mixed with chemokines, EPI and/or agonists as indicated, giving a final volume of 50 μl, for 10 s under orbital shaking conditions (1200 rpm) at 37°C on a commercial shaker (Eppendorf, Thermomixer R). The reactions were quenched with an equal volume of glutaraldehyde (1% final concentration) and platelet aggregation was determined by the loss of platelet singlets using a resistive-particle counter. This orbital shaking system gave similar amounts of platelet aggregation as seen in a test-tube, stirring system, with stir bars rotated at about 1000 rpm [11].

2.4. Treatment of platelets with Ptx

Experiments were carried out as described [21]. Washed platelets were preincubated at 37°C for 10 min and then for another 5 min in the presence of the ADP-ribosylation buffer, containing 15 μg/ml saponin, 0.2 mM NAD and 0.5 mM ATP, either alone (vehicle) or with PtxA protomer at 10 μg/ml. The resulting platelets were activated with agonists and aggregation determined as described above. Exposure to vehicle caused about 30% singlet loss, possibly resulting from the permeabilization procedure, causing leakage of compounds, including ADP, from the platelet cytosol.

2.5. Presentation of data

Data are shown as means ± S.E.M., from 3–4 experiments, and the Student's *t*-test was performed to determine whether two groups of experimental data were significantly different. Curve fitting was done by using a Logistic function provided by the Origin software from OriginLab Corporation (Northampton, MA, USA). IC_{50}s represent concentrations giving 50% inhibition from the expression $Y = 1/2(Y_{max} + Y_{min})$.

3. Results

3.1. EPI potentiates ADP-induced platelet aggregation

EPI enhances platelet aggregation induced by primary agonists including ADP and thrombin [14,22–24]. Therefore, we examined whether the effects of EPI on platelets were similar to those of the chemokines SDF-1, MDC and TARC [11]. EPI by itself induced little aggregation (< 5% above control). This was abolished when preparations were pretreated with the ADP scavenger apyrase (Fig. 1), suggesting that low ADP levels are present in the washed platelet preparations, as reported by earlier work [14].

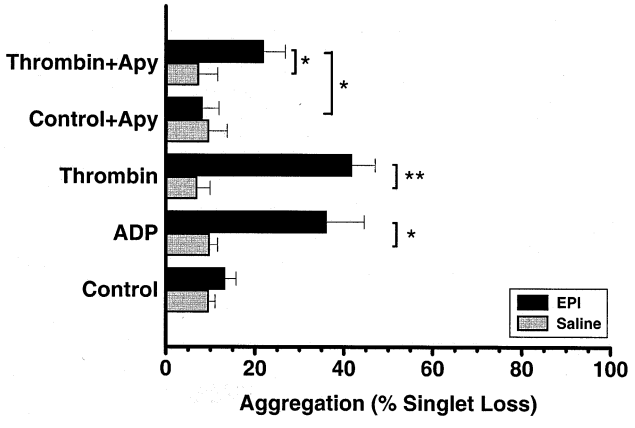

Fig. 1. Effects of EPI on platelet aggregation. Washed platelets were preincubated with saline or apyrase (Apy) and mixed for 10 s with saline, ADP (0.05 μM) or thrombin (0.01 U/ml), as indicated on the left, either alone (gray bars) or in combination with 15 μM EPI (black bars). Platelet aggregation was determined by the loss of platelet singlets (*$P < 0.05$; **$P < 0.01$).

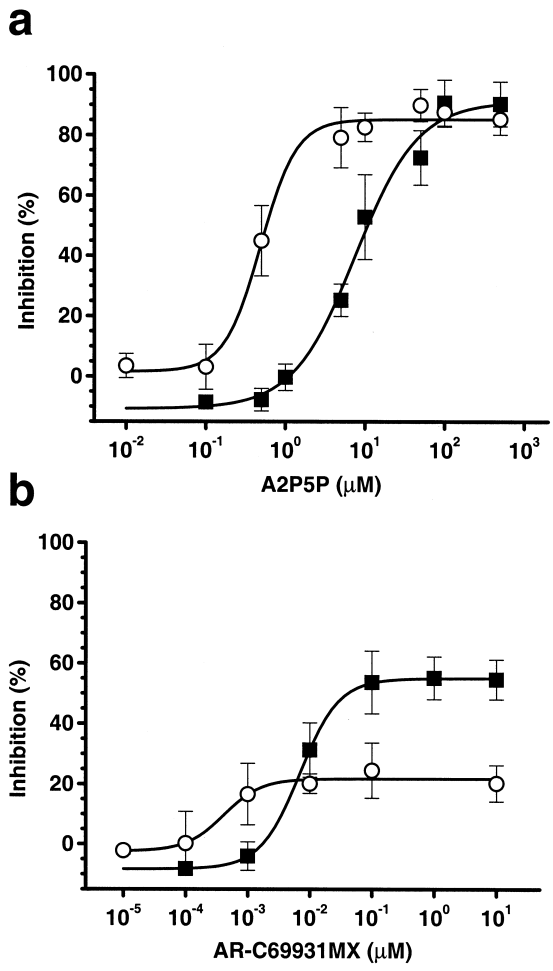

Fig. 2. Dose–response curves of ADP receptor antagonists A2P5P and AR-C69931MX. Washed platelets were preincubated with saline or the ADP antagonists A2P5P (a) or AR-C69931MX (b) and then challenged with 0.5 μM ADP (■) or 15 μM EPI+0.05 μM ADP (○). Platelet aggregation was assessed and the inhibition by the antagonists was calculated by setting aggregation induced by ADP (71.4 ± 0.9%) or EPI+ADP (46.7 ± 6.4%) as baseline aggregation with 0% inhibition.

Low levels of ADP (0.05 μM) and thrombin (0.01 U/ml) by themselves induced minimal aggregation (< 10%), compared to the control (9.7%, Fig. 1). EPI potentiated platelet activation by ADP and thrombin (36% and 42% aggregation, respectively), which for thrombin was partially inhibited by apyrase pretreatment. These effects of EPI on platelet aggregation and the influence of apyrase are similar to what we have recently observed for the chemokines SDF-1, MDC and TARC [11].

3.2. Dose-dependent inhibition of early platelet aggregation by ADP antagonists

The ADP receptor antagonists A2P5P and AR-C69931MX, specific for $P2Y_1$ and $P2T_{AC}$, respectively, were chosen [25,26], and we established dose–response curves for blocking the initial platelet aggregation induced by ADP alone (0.5 μM), which caused about 71% aggregation (Fig. 2a,b). A2P5P caused almost complete inhibition, with an IC_{50} of

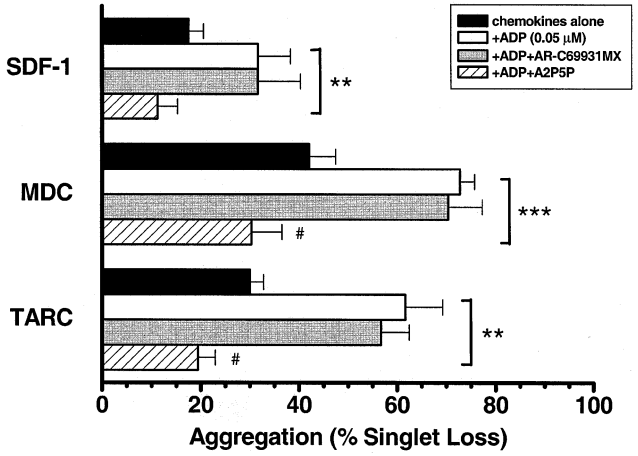

Fig. 3. Effects of A2P5P and AR-C69931MX on platelet aggregation induced by SDF-1, MDC and TARC. Washed platelets were preincubated with saline, A2P5P (100 μM) or AR-C69931MX (1 μM) and reacted with 0.5 μg/ml of SDF-1, MDC or TARC in combinations with ADP (0.05 μM). Platelet aggregation was determined as in Fig. 1 (**$P < 0.01$; ***$P < 0.005$; #$P < 0.05$, compared with 'saline control' in Fig. 1, $P < 0.05$).

6.7 μM. In contrast, AR-C69931MX was less effective, only causing about 55% inhibition and the IC_{50} was 6.9 nM. Next, we tested how the antagonists affected aggregation induced by a combination of EPI and a very low dose of ADP (0.05 μM). Such aggregation became very sensitive to the presence of A2P5P, with IC_{50} of 0.5 μM (Fig. 2a), but was resistant to inhibition by AR-C69931MX (Fig. 2b). Even though the IC_{50} was decreased (0.4 nM), the maximal inhibition was only about 20%.

3.3. Effects of ADP receptor antagonists on early platelet aggregation induced by the chemokines SDF-1, MDC and TARC

MDC and TARC by themselves caused platelet aggregation (42% and 30%, respectively), which was enhanced in the presence of low levels of ADP (0.05 μM, 72% and 62% aggregation, respectively, Fig. 3). However, SDF-1 was less effective, inducing only 18% aggregation by itself and 30% when combined with 0.05 μM ADP (Fig. 3). We then tested the receptor antagonists to evaluate which ADP receptors were responsible for the stimulating effect of the chemokines, choosing a dose of each which gave maximal inhibition (Fig. 2). AR-C69931MX did not inhibit early aggregation caused by SDF-1, MDC and TARC, while A2P5P diminished aggregation caused by SDF-1 down to levels which are similar to 'control saline' (Fig. 1). In contrast, A2P5P only partially blocked MDC- or TARC-induced aggregation (Fig. 3), to values still significantly above 'control saline' (Fig. 1, $P < 0.05$).

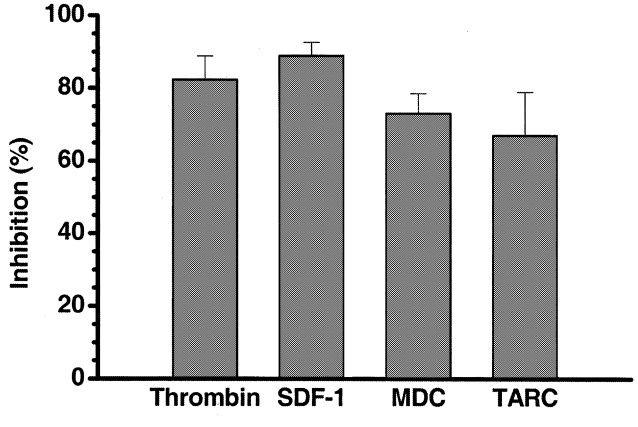

Fig. 4. Ptx inhibits SDF-1-, MDC- and TARC-induced platelet aggregation. Washed platelets were pretreated with vehicle (ADP-ribosylation buffer) or the vehicle containing 10 μg/ml Ptx for 5 min and mixed with 1 U/ml of thrombin or 0.5 μg/ml of SDF-1, MDC or TARC for 10 s. Platelet aggregation was calculated by setting platelet singlets in the vehicle or Ptx controls as 0% singlet loss, and inhibition was derived as in Fig. 2.

3.4. Ptx inhibits platelet aggregation induced by SDF-1, MDC and TARC

We next investigated whether chemokine-induced aggregation involves activation of $G_{\alpha i}$ proteins by using the bacterial toxin Ptx [21]. Exposure to Ptx strongly inhibited platelet aggregation induced by thrombin, SDF-1, MDC and TARC by 82.3%, 88.9%, 73.0% and 67.0%, respectively (Fig. 4).

4. Discussion

In the present study, we have investigated the synergism between low levels of ADP and the chemokines SDF-1, MDC and TARC in the early phase of platelet aggregation, using the ADP receptor antagonists A2P5P and AR-C69931MX specific to $P2Y_1$ and $P2T_{AC}$ receptors, respectively [25,26]. A2P5P strongly inhibited the stimulation caused by the chemokines, while AR-C69931MX had minimal effect (Figs. 2 and 3), and Ptx markedly suppressed the ability of SDF-1, MDC and TARC to stimulate platelet aggregation (Fig. 4).

EPI-enhanced platelet aggregation induced by low levels of ADP or thrombin was strongly decreased when the platelet preparations were pretreated with apyrase (Fig. 1), suggesting synergism between EPI, ADP and thrombin. Similar observations have been reported previously [13,14]. The inhibitory effects of A2P5P and AR-C69931MX on platelet aggregation caused by ADP and EPI were then examined. The aggregation induced by ADP (0.5 μM) was inhibited in a dose-dependent manner by the antagonists, with maximal inhibitions of 90% and 55% by A2P5P and AR-C69931MX, respectively. This result shows that A2P5P was much more potent than AR-C69931MX, suggesting that the activation of the ADP receptor $P2Y_1$ is involved during early aggregation (10 s) rather than that of $P2T_{AC}$. This finding is similar to the report showing that activation of $P2Y_1$ can induce primary aggregation without activation of $P2T_{AC}$ [18]. In addition, a combination of EPI with a low level of ADP, which by themselves caused minimal aggregation, resulted in aggregation that was markedly inhibited by A2P5P (85%), but resistant to AR-C69931MX (20% inhibition, Fig. 2a). As expected, the IC_{50} values for the antagonists were decreased by about 10-fold when the lower dose of ADP (0.05 μM) was used, instead of the higher dose of 0.5 μM. However, the maximal effect of AR-C69931MX was much less when EPI was present, decreasing from about 55% to 20% inhibition. These observations may be explained by the fact that EPI can also activate $G_{\alpha i}$, the G protein coupled to $P2T_{AC}$, such that when $P2T_{AC}$ is blocked by the ADP receptor antagonist, EPI can partially bypass the 'block'.

Similar to EPI, aggregation caused by SDF-1, a ligand for CXCR4, was completely prevented by the $P2Y_1$ antagonist A2P5P which blocks activation of the $G_{\alpha q}$ protein by ADP [26]. Recent studies suggest that coactivation of both $G_{\alpha i}$ and $G_{\alpha q}$ proteins is required for effective platelet aggregation [15,27]. Therefore, the complete inhibition of SDF-1 by A2P5P is consistent with the inability of SDF-1 to stimulate $G_{\alpha q}$ signalling pathways. This is supported by observations in our laboratory [11] and by Kowalska et al. [9], who reported that SDF-1 by itself fails to mobilize calcium, which reflects activation of phospholipase C by $G_{\alpha q}$ and $G_{\beta\gamma}$ proteins [28,29].

In contrast, A2P5P only partially inhibited aggregation induced by MDC and TARC, ligands for CCR4 (Fig. 3). Under such conditions, activation of $G_{\alpha q}$ by ADP was blocked, suggesting that MDC and TARC by themselves can activate $G_{\alpha q}$-dependent signalling, supported by recent evidence that MDC induces calcium mobilization and may activate $G_{\alpha q}$ [9,11].

As with EPI, early platelet aggregation stimulated by SDF-1, MDC and TARC was resistant to the treatment with the $P2T_{AC}$ antagonist AR-C69931MX (Fig. 3). Since EPI activates a $G_{\alpha i}$-coupled receptor, we hypothesized that the 'resistance' of the chemokines to the antagonist may be because they also stimulate $G_{\alpha i}$. The experiments using Ptx, a $G_{\alpha i}$ inhibitor, were carried out to test this hypothesis. As expected, Ptx strongly inhibited aggregation induced by SDF-1, MDC or TARC. This agrees with recent reported experiments showing inhibition of SDF-1-induced aggregation by the toxin [10]. These results suggest that the effects of the chemokines involve Ptx-sensitive $G_{\alpha i}$ proteins.

In conclusion, we believe that the $P2Y_1$ ADP receptor is particularly important for activating events leading to rapid platelet aggregation (10 s), rather than the $P2T_{AC}$ receptor. $P2Y_1$ is also essential for the ability of the chemokines SDF-1, MDC and TARC to initiate aggregation, which involves the activation of Ptx-sensitive $G_{\alpha i}$ proteins. However, it is important to note that the $P2T_{AC}$ receptor may play a greater role in the later sustained aggregation response as has been demonstrated for ADP [18]. Our observations suggest that SDF-1 only activates $G_{\alpha i}$ proteins, while MDC and TARC stimulate not only $G_{\alpha i}$, but also $G_{\alpha q}$-dependent signalling pathways. Further experiments will be required to understand fully the mechanisms of platelet activation by these chemokines, including the relative roles of α and $\beta\gamma$ G protein subunits.

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