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SPECIFIC BINDING OF THE THROMBOXANE A_2 ANTAGONIST 13-AZAPROSTANOIC ACID TO HUMAN PLATELET MEMBRANES

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In the present study we characterized the interaction between the thromboxane A_2/prostaglandin H_2 antagonist, *trans*-13-azaprostanoic acid (13-APA), and isolated human platelet membranes. In these studies, we developed a binding assay using *trans* [^{3}H] 13-APA as the ligand. It was found that *trans* [^{3}H] 13-APA specific binding was rapid, reversible, saturable and temperature dependent. Scatchard analysis of the binding data yielded a curvilinear plot which indicated the existence of two classes of binding sites: a high-affinity binding site with an estimated dissociation constant (K_d) of 100 nM; and a low-affinity binding site with an estimated K_d of 3.5 μM. At saturation, approximately 1 pmol/mg protein of [^{3}H] 13-APA was bound to the high affinity site. In order to further characterize the nature of the [^{3}H] 13-APA binding site, we evaluated competitive binding by *cis* 13-APA, *cis* 15-APA, prostaglandin $F_{2\alpha}$, U46619, 6-ketoprostaglandin $F_{1\alpha}$ and thromboxane B_2. It was found that the [^{3}H] 13-APA binding site was stereospecific and structurally specific. Thus, the *cis* isomer of 13-APA exhibited substantially reduced affinity for binding. Furthermore, the prostaglandin derivatives, thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$, which do not possess biological activity, also did not compete for [^{3}H] 13-APA binding. On the other hand, U46619 which acts as a thromboxane A_2/prostaglandin H_2 mimetic, and prostaglandin $F_{2\alpha}$ which acts as a thromboxane A_2/prostaglandin H_2 antagonist, both effectively competed for [^{3}H] 13-APA binding. These findings indicate that *trans* 13-APA binds to a specific site on the platelet membrane which presumably represents the thromboxane A_2/prostaglandin H_2 receptor.

Introduction

Trans 13-azaprostanoic acid, 13-APA, has been previously shown to specifically and competitively inhibit human platelet aggregation mediated through the arachidonic acid cascade [1–3]. Its mechanism of action does not involve inhibition of cyclooxygenase or thromboxane synthetase. Rather, 13-APA directly antagonizes prostaglandin H_2 and/or thromboxane A_2. Furthermore, 13-APA neither stimulates cAMP production nor alters increases in cAMP stimulated by prostacyclin or prostaglandin E_1 [1]. These characteristics indicate that 13-APA specifically interferes with platelet activation induced by thromboxane A_2/prostaglandin H_2. Evidence that this antagonism is mediated through a receptor interaction is provided by the following observations [1,3]: (1) evaluation of a series of azaprostanoids demonstrates that the inhibitory properties are markedly dependent upon the integrity of the normal 20-carbon prostaglandin skeletal arrangement, i.e., homo-13-APA and nor-13-APA exhibit decreased activity; (2) the inhibitory properties are sensitive to the location of the nitrogen in the

Abbreviation: APA, azaprostanoic acid.

molecule, i.e., 15-APA and 7-APA exhibit decreased activity; (3) the inhibitory properties are stereospecific, i.e., *trans* 13-APA is significantly more potent than *cis* 13-APA; (4) the inhibitory properties of 13-APA are competitive and reversible; and (5) 13-APA selectively antagonizes thromboxane A_2/prostaglandin H_2 in other organ systems, i.e. the rat aorta and the canine mesenteric artery [4].

In order to gain additional information regarding the interaction of 13-APA with human platelets, we evaluated the binding of *trans* [^{3}H] 13-APA to isolated platelet membranes. It was found that [^{3}H] 13-APA specifically binds to platelet membranes and the binding is temperature dependent, reversible, saturable and stereospecific.

Materials and Methods

Human blood anticoagulated with citrate-phosphate-dextrose adenine solution (CPD-A1; 0.38%) was obtained from apparently healthy donors who had not taken any drugs for 10 days prior to donation. Platelet-rich plasma was prepared by centrifugation of the whole blood at $164 \times g$ for 15 min. Platelet aggregation and secretion were measured simultaneously using a Lumi-Aggregometer (Chrono-Log) [5].

Platelet membranes were prepared according to the following procedure: approx. 70 ml of human platelet-rich plasma was centrifuged at $4000 \times g$ for 10 min at 4°C to pellet the platelets. In order to remove the residual plasma protein, the platelet pellet was washed once with 30 ml of buffer containing 25 mM Tris-HCl/5 mM $MgCl_2$ (pH 7.4) and recentrifuged to pellet the platelets. The platelets were then resuspended in 20 ml of the same buffer, and the cells were disrupted by sonication. The sonication was performed on ice using a Kontes Sonicator (Vineland, NJ). The platelets were sonicated for a total 90 s with a 15 s burst followed by a 15 s intermission. The sonicated mixture was centrifuged at $100\,000 \times g$ for 1 h and the membrane fraction was suspended in the same buffer. The protein concentration was determined by the method of Lowry et al. [6] and then adjusted to 3 mg/ml.

The binding assay was performed at 37°C. One ml of the resuspended membrane fraction was incubated with 0.5 nmol of [^{3}H] 13-APA. Four (0.1 ml) aliquots of the incubation mixture were filtered at each timepoint through a glass fiber membrane (GF/C, Whatman) to separate the membrane bound from free [^{3}H] 13-APA. The membranes were quickly washed twice with 5 ml of cold buffer, mixed with 10 ml ACS scintillation fluid (Amersham) and counted for [^{3}H] 13-APA. The specific binding of [^{3}H] 13-APA was determined by incubating the membrane fractions with [^{3}H] 13-APA for 20 min followed by addition of 1000-fold molar excess of unlabelled 13-APA or other prostaglandin-like derivatives. Scatchard analysis [7,8] was performed by incubating the membrane fraction with various concentrations of [^{3}H] 13-APA. Specific binding was calculated by subtracting the binding of [^{3}H] 13-APA (in the presence of 1000-fold excess of unlabelled 13-APA) from total [^{3}H] 13-APA binding.

[^{3}H] 13-APA (44 Ci/mmol) was synthesized by catalytic reduction of the amino-olefin precursor (unpublished results). The purity of [^{3}H] 13-APA was determined by reverse phase TLC (LKC18, Whatman) and developed with a solvent system containing water/methanol (44 : 56, v/v) and 60 mM ammonium acetate. A single spot with an estimated R_F of 0.2 was visualized by spraying the plate with sodium nitroprusside. Approx. 98% of the radioactivity applied to the plate was recovered within the spot.

Adenosine 5′-triphosphate (monosodium) and arachidonic acid were purchased from Sigma Chemical Co., St. Louis, MO. Prostaglandin $F_{2\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$, thromboxane B_2 and U46619 were kindly supplied by the Upjohn Co., Kalamazoo, MI. *Trans* 13-APA, *cis* 13-APA and *cis* 15-APA were synthesized as previously described [3].

Results

Effect of 13-APA on human platelet aggregation and secretion

It can be seen in Fig. 1 that 30 μM 13-APA completely inhibited 500 μM arachidonic acid-induced aggregation and secretion, whereas 13-APA had no effect on 10 μM ADP or 5 μM A23187-induced aggregation, even when the concentration of 13-APA was increased to 150 μM.

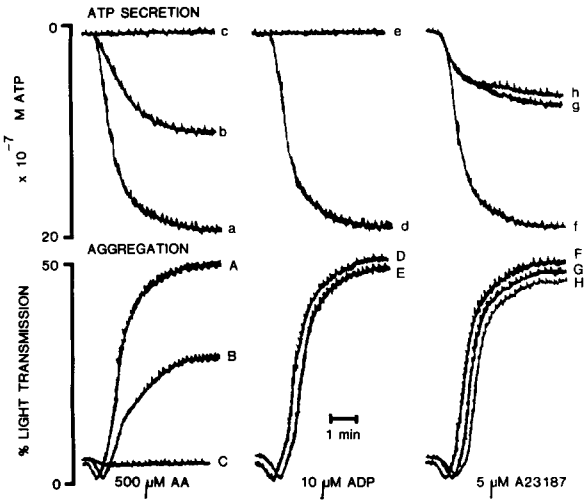

Fig. 1. Effect of 13-azaprostanoic acid (13-APA) on human platelet aggregation and ATP secretion. Each aggregating agent was titrated to produce maximal aggregation. The platelet-rich plasma was pretreated with various concentrations of 13-APA for 2 min followed by addition of aggregating agent. Platelet aggregation and secretion were monitored simultaneously using a Lumi-Aggregometer. 500 μM arachidonic acid (AA): 13-APA vehicle (A,a); 15 μM 13-APA (B,b); 30 μM 13-APA (C,c). 10 μM ADP: 13-APA vehicle (D,d); 100 μM 13-APA (E,e). 5 μM A23187: 13-APA vehicle (F,f); 150 μM 13-APA (G,g); 20 μM Indomethacin (H,h).

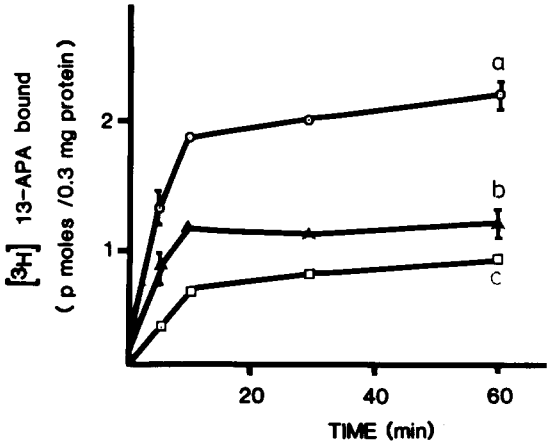

Fig. 2. Time course of [^{3}H] 13-APA binding to isolated human platelet membranes. Total binding was determined by adding 0.5 nmoles of [^{3}H] 13-APA to each ml of membrane preparation containing 3 mg membrane protein. Nonspecific binding was determined by adding 1000-fold excess of unlabeled 13-APA prior to [^{3}H] 13-APA. The specific binding was calculated by subtracting nonspecific binding from total [^{3}H] 13-APA binding. Values represent the mean ± S.E. from at least three separate experiments. a (○), [^{3}H] 13-APA total binding; b (▲), [^{3}H] 13-APA + 1000-fold excess 13-APA; c (□), specific binding.

On the other hand, ADP-induced secretion of ATP was completely blocked by 30 μM 13-APA. Furthermore, 30 μM 13-APA also inhibited A23187-induced ATP secretion to the same extent as observed with 20 μM indomethacin treatment.

Characteristics of [^{3}H] 13-APA binding to the platelet membrane fraction

When 0.5 nmol of [^{3}H] 13-APA was incubated with 1 ml of the platelet membrane fraction, filtered through a glass fiber membrane and washed with cold buffer, a substantial amount of radioactivity, i.e., typically 15000 cpm/mg protein, remained associated with the platelet membranes. The time course of [^{3}H] 13-APA binding at 37°C is shown in Fig. 2 (top trace). As can be seen, the total binding reached equilibrium within 10 min and remained at approximately the same level for the next 50 min. When 1000-fold molar excess of unlabelled 13-APA was preincubated with the membrane fraction prior to the addition of [^{3}H] 13-APA, the total binding of [^{3}H] 13-APA was inhibited by approx. 40% (Fig. 2, middle trace). Fig. 2 (lower trace) illustrates the difference between total binding (top trace) and nonspecific binding (middle trace) and represents specific binding to the platelet membranes. A similar result was obtained in displacement studies. Thus, when the same excess of unlabelled 13-APA was added after the binding of [^{3}H] 13-APA had reached equilibrium, approx. 40% of the total binding was displaced within 20 min (Fig. 3). Increasing the incubation time beyond 20 min did not significantly change the extent of displacement.

In order to determine the effect of temperature on the binding of [^{3}H] 13-APA to the platelet membranes, three different temperatures, i.e., 4°C, 25°C and 37°C were examined (Fig. 4). It was found that the maximal binding of [^{3}H] 13-APA to specific binding sites was obtained at 37°C (Fig. 4, upper trace). Lowering the temperature to 25°C did not significantly reduce [^{3}H] 13-APA specific binding (Fig. 4, middle trace). However, when the incubation was carried out at 4°C, a substantial reduction in the specific binding was observed (Fig. 4, lower trace).

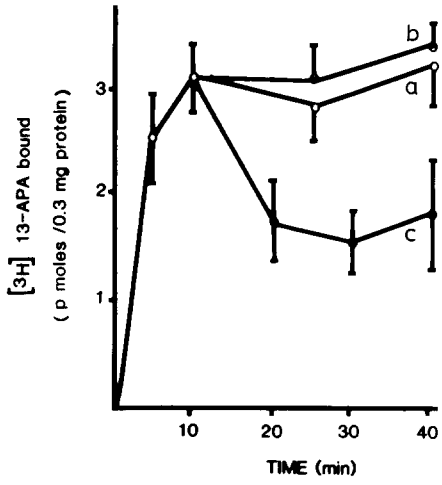

Fig. 3. Reversible binding of [^{3}H] 13-APA to isolated platelet membranes. Each ml of platelet membrane suspension was incubated with 0.5 nmoles of [^{3}H] 13-APA at 37°C until equilibrium was reached. 1000-fold molar excess of unlabelled 13-APA or thromboxane B_2 was then added to the incubation mixture. The binding of [^{3}H] 13-APA was determined as described in Methods. (a) Plus vehicle; (b) plus thromboxane B_2; (c) plus 13-APA.

The saturation curve for [^{3}H] 13-APA specific binding is illustrated in Fig. 5. It can be seen that the specific binding sites were saturated at 3 μM 13-APA. Scatchard analysis (Fig. 5B) of this binding data yielded a curvilinear plot indicating that there are two classes of binding sites. A high-affinity binding site with an estimated K_d of 100 nM bound approx. 1 pmol of [^{3}H] 13-APA per mg of membrane protein; and a low-affinity binding site with an estimated K_d of 3.5 μM bound approx. 10 pmol of [^{3}H] 13-APA per mg of membrane protein.

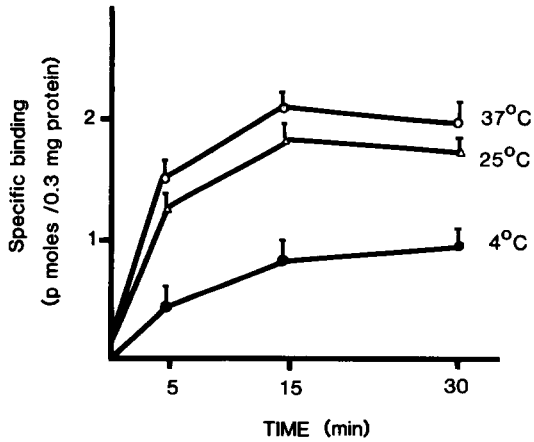

Fig. 4. Temperature dependence of [^{3}H] 13-APA binding. [^{3}H] 13-APA binding was determined at 37°C, 25°C and 4°C by the procedure described in Methods. Values represent the mean ± S.E. from three separate experiments.

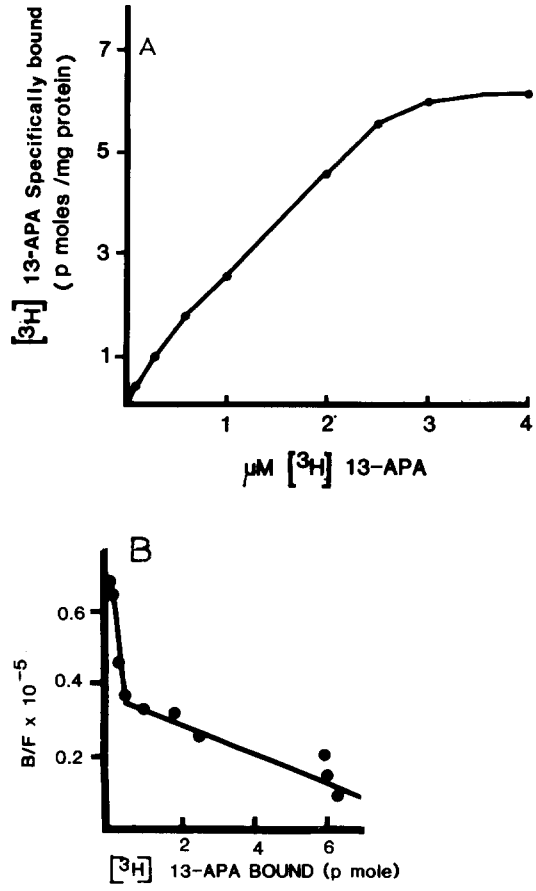

Fig. 5. A. Saturation binding of [^{3}H] 13-APA. Platelet membranes were incubated with various concentrations of [^{3}H] 13-APA until equilibrium binding was reached (20 min). B. Scatchard analysis of the [^{3}H] 13-APA binding, transformed from the saturation binding curve.

Specificity of [^{3}H] 13-APA binding

Previous aggregation studies [3] indicated that the inhibitory potency of a series of azaprostanoids is dependent upon the integrity of the natural prostaglandin molecular structure and the location of the nitrogen atom within the molecule. Slight deviation from this natural skeletal arrangement

resulted in decreased biological activity. Thus, *trans* 13-APA was found to be a more potent inhibitor of arachidonic acid-induced aggregation than either *cis* 13-APA or *cis* 15-APA. In order to determine whether this structure-activity relationship for aggregation is reflected in the binding affinity, we examined the relative ability of these two azaprostanoids, i.e., *cis* 13-APA and *cis* 15-APA to compete with *trans* [^{3}H] 13-APA binding. It was found (Fig. 6, traces D and E) that 1000-fold molar excess of *cis* 13-APA or *cis* 15-APA displaced only 60% or 55%, respectively, of the *trans* [^{3}H] 13-APA specific binding. This finding indicates a lesser affinity of these azaprostanoids for the *trans* 13-APA binding sites and is in agreement with our previous aggregation results.

We next examined the ability of 9,11-methanoepoxy-prostaglandin H_2 (U46619) to displace [^{3}H] 13-APA binding. U46619 is known to mimic the action of thromboxane A_2/prostaglandin H_2 presumably by interacting with the thromboxane A_2/prostaglandin H_2 receptor site [9]. If 13-APA also binds to this putative receptor, it would be expected that U46619 should compete for [^{3}H] 13-APA binding. This was indeed found to be the case. It can be seen (Fig. 6, trace B) that

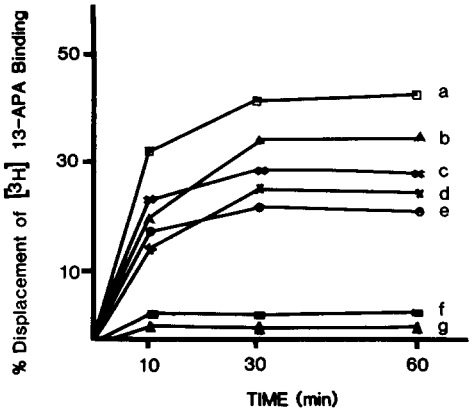

Fig. 6. Displacement of *trans* [^{3}H] 13-APA binding. Platelet membranes were incubated with 0.5 nmoles [^{3}H] 13-APA/ml until equilibrium binding was achieved (20 min). Unlabelled *trans* 13-APA, *cis* 13-APA, *cis* 15-APA, U46619, prostaglandin $F_{2\alpha}$, thromboxane B_2 or 6-ketoprostaglandin $F_{1\alpha}$ was then added at 1000-fold molar excess. Values represent the percent displacement of total [^{3}H] 13-APA binding. a, 13-APA (41 ± 6); b, U46619 (34 ± 6); c, prostaglandin $F_{2\alpha}$ (29 ± 5); d, *cis* 13-APA (28 ± 4); e, *cis* 15-APA (26 ± 4); f, thromboxane B_2 (3 ± 5); g, 6-ketoprostaglandin $F_{1\alpha}$ (3 ± 3).

U46619 at 1000-fold molar excess displaced approx. 80% of [^{3}H] 13-APA specific binding within 30 min of incubation. In similar experiments we evaluated the ability of prostaglandin $F_{2\alpha}$ to compete for 13-APA binding sites. Previous studies have suggested that prostaglandin $F_{2\alpha}$ specifically inhibits arachidonic acid- or U46619-induced platelet aggregation by antagonism at the thromboxane A_2/prostaglandin H_2 receptor level [10,11]. Consistent with this notion, excess prostaglandin $F_{2\alpha}$ caused a 70% displacement of [^{3}H] 13-APA specific binding as shown in Fig. 6 (trace C).

In order to further characterize the specificity of [^{3}H] 13-APA binding, thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ (which exhibit no inhibitory effects on arachidonic acid- or U46619-induced aggregation) were tested for their abilities to displace [^{3}H] 13-APA specific binding. It was found (Fig. 6, traces F and G) that excess thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ did not significantly reduce the binding of [^{3}H] 13-APA.

Discussion

In the last few years significant progress has been made in understanding the inhibitory mechanisms of certain prostaglandins on platelet function. In this respect, it has been demonstrated that prostacyclin [12–14], prostaglandin E_1 [15] and prostaglandin D_2 [16,17] are powerful adenylate cyclase stimulators which mediate their effects by presumably binding to receptors on the platelet membrane. In contrast, the interaction of the stimulatory arachidonic acid metabolite, i.e. thromboxane A_2, with the platelet has not been characterized. This is primarily due to the molecular nature of thromboxane A_2 which exhibits a half-life of approx. 30 s at physiological temperature and pH. Consequently, it has not been possible to isolate and purify thromboxane A_2 in sufficient amounts for biological evaluations. Initially, experiments examining the stimulatory activity of thromboxane A_2 were limited to indirect measurements using various inhibitors of thromboxane A_2 synthesis, i.e., cyclooxygenase blockers and thromboxane A_2 synthetase blockers [18–25]. More recently, however, several stable compounds have been synthesized which directly antagonize the action of thromboxane A_2, e.g. 9,11-epoxyimino-

prosta-5,13-dienoic acid (9,11-EIP) [26], carbocyclic thromboxane A_2 [27], pinane-thromboxane A_2 [28], 9,11-azo-13-oxa-15-hydroxyprostanoic acid [29], bisenoic prostaglandins [30], 13-azaprostanoic acid [3], etc. Among these antagonists, 13-APA appears to possess the most specific properties in that 13-APA inhibits thromboxane A_2 activation of both the platelet and the vasculature but does not interfere with the metabolism of arachidonic acid [1–4]. Furthermore, our previous results demonstrate that inhibition of arachidonic acid-induced aggregation by azaprostanoids exhibits stereospecificity, structural specificity and reversibility. Finally, 13-APA does not cause cAMP accumulation nor is it involved in the stimulation of adenylate cyclase by prostaglandin E_1 or prostaglandin I_2.

In the present studies we further characterized the interaction between 13-APA and platelets by measuring [^{3}H] 13-APA binding to the isolated platelet membrane. It was found that the specific binding of [^{3}H] 13-APA was rapid, reversible, saturable and temperature dependent. All of these characteristics are consistent with the notion that 13-APA interacts with a physiologically relevant platelet receptor site. In this regard, the observed time course for 13-APA binding is comparable to that observed for other prostanoids, e.g., prostacyclin [14] or prostaglandin E_1 [15]. The reversibility of [^{3}H] 13-APA specific binding is also consistent with our previous observation that arachidonic acid-induced aggregation can be reversed by 13-APA [1,2]. Finally, the saturability and temperature dependence further implicate a specific 13-APA-receptor interaction on the platelet membrane.

Scatchard analysis of [^{3}H] 13-APA binding yielded two classes of binding sites with different affinity constants. It is not clear whether these two classes of binding sites are associated with negative cooperativity within one single class of binding sites or truly reflected the presence of heterogeneity of binding sites. In either case, the high-affinity binding of [^{3}H] 13-APA, with an apparent dissociation constant (K_d) of 100 nM, is presumably responsible for the observed biological response. On the other hand, the apparent K_d of the high-affinity binding sites (100 nM) was found to be approximately two orders of magnitude lower than the concentration of 13-APA required for 50% inhibition of aggregation (IC_{50}). The disagreement between the K_d for binding and the IC_{50} for aggregation could result from different experimental conditions employed in each study. The K_d of 13-APA was determined by using a relatively pure membrane fraction and reflected a direct physical interaction of 13-APA with its receptor, whereas the concentration of 13-APA required for 50% inhibition aggregation reflected a complex interaction between plasma protein, the agonist, the antagonist and the platelets. We found a similar discrepancy between the K_d for binding and the IC_{50} for aggregation with prostaglandin $F_{2\alpha}$ [10,11]. In these studies, the K_d for [^{3}H] prostaglandin $F_{2\alpha}$ was also approximately two orders of magnitude lower than the IC_{50} for aggregation.

The competitive binding experiments with different azaprostanoids demonstrated that *cis* 13-APA and *cis* 15-APA only partially displaced *trans* [^{3}H] 13-APA specific binding (Fig. 6). Furthermore, since thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ did not significantly reduce the binding of [^{3}H] 13-APA, the observed displacement of binding by either *cis* azaprostanoid must be associated with specific [^{3}H] 13-APA binding sites. In addition, since the *cis* azaprostanoids displaced less [^{3}H] 13-APA binding than unlabelled *trans* 13-APA, the binding affinity of the *cis* isomers is presumably lower than that of *trans* 13-APA. Finally, the binding affinity of the azaprostanoids appears to agree closely with the inhibitory activity obtained in aggregation studies [3], suggesting that there is a relationship between binding affinity and biological potency. A similar relationship has been observed in the prostaglandin-receptor system of frog erythrocyte membranes and human blood platelets [31,15,16].

Based on the above findings, it would appear that the relative ability of an agent to compete with [^{3}H] 13-APA binding could be used as a parameter to determine whether or not the agent acts at the thromboxane A_2 receptor. Thus, an agent which mediates its effects through thromboxane A_2-receptor interaction would be expected to displace [^{3}H] 13-APA specific binding to an extent dependent upon the potency of that particular agent. In this regard, we examined the ability of the thromboxane A_2 antagonist, prostaglandin $F_{2\alpha}$

[11] and the thromboxane A_2 mimetic, U46619 [9] to displace [^{3}H] 13-APA binding. It was found that prostaglandin $F_{2\alpha}$ and U46619 were capable of competing with [^{3}H] 13-APA binding to an extent which was nearly equal to that observed using unlabelled 13-APA. These findings are consistent with our previous report [11] in which approx. 85% of the ^{3}H-labeled prostaglandin $F_{2\alpha}$ specific binding was displaced by either 13-APA or U46619. These results also suggest that prostaglandin $F_{2\alpha}$, U46619 and 13-APA all mediate their effects by binding to the same receptor site.

Evidence supporting the specificity of the 13-APA-receptor interaction was provided by aggregation and secretion studies using arachidonic acid, ADP or the divalent cation ionophore A23187. It was found that the concentrations of 13-APA which completely blocked arachidonic acid-induced aggregation had no effect on primary aggregation induced by ADP or A23187. On the other hand, platelet secretion, which is known to be mediated by thromboxane A_2, was suppressed by 13-APA in all cases.

In summary, this study provides direct evidence that thromboxane A_2 causes platelet activation by interacting with a specific receptor, and that 13-APA blocks thromboxane A_2-stimulated activation through competition for that receptor site. Although it is not known how thromboxane A_2-receptor interaction leads to platelet activation, recent results have demonstrated that thromboxane A_2 and/or prostaglandin H_2 can cause Ca^{2+} release from isolated platelet membrane vesicles [32,33]. Furthermore, this Ca^{2+} release in response to thromboxane A_2/prostaglandin H_2 is completely blocked by 13-APA [33]. This finding suggests that the binding of thromboxane A_2/prostaglandin H_2 to the putative receptor is the initiating event in the Ca^{2+} release process.

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