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Binding of an ^{125}I-Labeled Thromboxane A_2/Prostaglandin H_2 Receptor Antagonist to Washed Canine Platelets

DALE E. MAIS, PAMELA J. KOCHEL, DAVID L. SAUSSY, JR., AND PERRY V. HALUSHKA[1]

Departments of Pharmacology (D.E.M., P.J.K., D.L.S., P.V.H.) and Medicine (P.V.H.), Medical University of South Carolina, and The Drug Science Foundation (D.E.M.), Charleston, South Carolina 29425

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SUMMARY

A binding site for the thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) antagonist ^{125}I-PTA-OH (9,11-dimethylmethano-11,12-methano-16-(4-methoxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2) to washed canine platelets is described. ^{127}I-PTA-OH competitively antagonized aggregation induced by the TXA_2/PGH_2 mimetic U46619. A Schild analysis of the pharmacologic study revealed pA_2 of 7.97 and a slope of −0.95. The pA_2 value yielded a K_d of 11 nM. Specific binding in Tris-NaCl buffer (pH 7.4) is not affected by extracellular Ca^{2+} or Mg^{2+} in concentrations up to 750 μM. The pH optimum for binding resides between 7.0 and 7.4. The association rate constant, k_1, was 4.5×10^6 M^{-1} min^{-1}, and the dissociation rate constant, k_{-1}, was 1.45×10^{-1} min^{-1}, yielding a kinetically determined K_d (k_{-1}/k_1) of 32 nM. Scatchard analysis of I-PTA-OH binding to washed canine platelets revealed two classes of binding sites, a high affinity site (K_d = 24 nM, B_{max} = 71 fmol/10^7 platelets) (4400 binding sites/platelet) and a low affinity site (K_d = 2.1 μM). Several TXA_2/PGH_2 receptor antagonists competed with specific ^{125}I-PTA-OH binding, and the rank order of potency for displacing the ligand correlated (r = 0.97) with the rank order of potency for their ability to inhibit U46619-induced aggregation in canine platelet-rich plasma. Prostaglandins $F_{2\alpha}$ and E_2 also displaced the ligand, but only at much higher concentrations. Binding of I-PTA-OH or the TXA_2/PGH_2 mimetic U46619 was unaffected by the aggregating agents epinephrine (10 μM) or ADP (5 μM). The similarity in the K_d values obtained kinetically, by equilibrium binding studies for the high affinity site and by Schild analysis, suggests that this high affinity site mediates TXA_2/PGH_2 induced platelet aggregation. In addition, the close correlation between the abilities of the antagonists to displace the ligand and to inhibit U46619-induced aggregation suggests that this site may represent a TXA_2/PGH_2 receptor.

INTRODUCTION

The metabolism of arachidonic acid by platelets results in the formation of the prostaglandin endoperoxides, PGG_2[2] and PGH_2 (1), and TXA_2 (2). Initial studies of these compounds were descriptive in nature and found them to be potent vasoconstrictors and stimulators of platelet aggregation (1–4). More recently, the synthesis of stable analogs of the endoperoxides and TXA_2, which act as either mimetics of or antagonists to the actions of the naturally occurring eicosanoids, has provided the tools for investigations into the mechanisms of action of these compounds and the nature of their receptors (5–8).

Pharmacologic studies with a series of receptor antagonists and agonists have provided evidence for the existence of distinct classes of TXA_2/PGH_2 receptors in platelets and blood vessels (9). While the pharmacologic evidence is suggestive for the existence of TXA_2/PGH_2 receptors in vessels and human platelets, studies by previous workers suggest that these receptors may not exist in canine platelets (10). Canine platelets are normally unresponsive to arachidonic acid or stable TXA_2 mimetics. This unresponsiveness is reversed if the platelets are preincubated with subthreshold levels of epinephrine. The effect of epinephrine may be to alter intraplatelet cAMP levels, as was previously suggested (10), which could lower the threshold for another aggregatory stimulus. Alternatively, it may alter a platelet TXA_2/PGH_2 receptor via changing the affinity for the agonist and/or receptor density. Radioligand binding studies using the appropriate ligands may help to clarify these possibilities.

This paper reports the radioligand binding studies which have been carried out using the ^{125}I-labeled 13-

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[2] The abbreviations used are: PG, prostaglandin; TXA_2, thromboxane A_2.

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azapinane-TXA_2 derivative ^{125}I-PTA-OH in washed canine platelets.

EXPERIMENTAL PROCEDURES

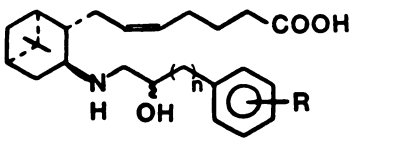
Materials. Fig. 1 shows the structures of the antagonists used in these studies. The synthesis and characterization of PTA-OH (9,11-dimethylmethano-11,12-methano-16-(4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2), PTA-OM (9,11-dimethylmethano-11,12-methano-16-(4-methoxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2), ^{127}I-PTA-OH (9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2), $^{127}I_2$-PTA-OH (9,11-dimethylmethano-11,12-methano-16-(3,5 diiodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2), and PTA-(ω-1) (9,11-dimethylmethano-11,12-methano-15-phenyl-13,14-dihydro-13-aza-15$\alpha\beta$-ω-pentanor-TXA_2) were carried out as described previously (8). All the compounds are substituted analogs of ONO-11120 except PTA-(ω-1) which is the methylene-contracted analog of ONO-11120. ONO-11120 (9,11-dimethylmethano-11,12-methano-16-phenyl-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2) was a gift from ONO Pharmaceutical Company (Osaka, Japan). U46619 (15*S*-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid) was a gift from The Upjohn Co, and PGE_2 and $PGF_{2\alpha}$ were purchased from The Upjohn Co. All other reagents were of the highest purity available from Sigma.

Iodination (^{125}I) of PTA-OH. A stock solution of PTA-OH (1 μg/μl) was prepared by dissolving it in absolute ethanol. Under these conditions, the compound was stable at −20° for several months. For the iodination procedure, the stock solution was diluted to a concentration of 0.05 μg/μl in 0.1 M phosphate buffer (pH 7.4). The iodination was carried out in a 1-ml Reacti Vial. The following substances were added in the order given: (5 μl) 0.1 M phosphate buffer (pH 7.4), (10 μl) PTA-OH (0.05 μg/μl), 0.5 mCi of Na^{125}I (Amersham Corp.) (5 μl), and (10 μl) chloramine T (50 μg/ml). The vial was vortexed for several seconds and then allowed to stand at room temperature for 75 sec, after which 10 μl of sodium metabisulfite (30 mg/ml) was added and vortexed for an additional 5 sec. If incubation times of longer than 75 sec were used, diiodo-PTA-OH (I_2-PTA-OH) began to form in addition to other products which decreased the specific binding. This technique could be utilized in the synthesis of ^{125}I-PTA-OH using quantities of up to 4 mCi of Na^{125}I with corresponding increases in the quantities of other reaction components.

The reaction mixture was applied to a silica gel thin layer chromatography (250 μm thick) plate (Fisher), except for approximately 1 μl which was applied to a second plate with 10 μg each of ^{127}I-PTA-OH, PTA-OH, and I_2-PTA-OH. The solvent system used was: *n*-hexane:glacial acetic acid:ethyl acetate:H_2O (16:18:52:60). The organic layer of the two phases was used for developing the plates. After development to approximately 10 cm, the second plate was dried and scanned in a Packard radiochromatogram scanner. A representative tracing is shown in Fig. 2. The zone corresponding to the ^{125}I-PTA-OH on the radiochromatogram was marked on the first plate, and the silica was scraped into a glass conical test tube containing 5 ml of chloroform:methanol (9:1). The mixture was vortexed for 1 min and then centrifuged to separate the layers. The organic layer was aspirated and retained, and the procedure was repeated twice more. The $CHCl_3$:MeOH layers containing ^{125}I-PTA-OH were pooled and stored for up to 2 months at −20°. Approximately 20% of the Na^{125}I was recovered as ^{125}I-PTA-OH. Trying to increase the incorporation of ^{125}I into PTA-OH using several different approaches led to problems with the ^{125}I-PTA-OH having a much greater nonspecific binding compared to that of the isotope made by the described procedure. The R_F value of the ^{125}I-PTA-OH was identical to that of ^{127}I-PTA-OH. Since ^{125}I-PTA-OH was completely separated from PTA-OH and I_2-PTA-OH, the final specific activity was taken to be that of the I^{125}Na.

The radiolabeled ligand was prepared freshly on the day of use in the following manner. An aliquot of the $CHCl_3$:MeOH solution was placed into a glass tube (12 × 75 mm) and dried under a stream of nitrogen at room temperature. The ligand was redissolved in the appropriate volume of assay buffer (50 mM Tris, 100 mM NaCl, 5 mM dextrose, and 10 μM indomethacin, (pH 7.4) with vortex mixing for 1 min.

Platelet aggregation. Blood (50–100 ml) was drawn from mongrel dogs of either sex after pentobarbital (30 mg/kg) anesthesia into syringes containing indomethacin (10 μM) and EDTA (5 mM) (final concentrations). The blood was centrifuged at 100 × *g* for 15 min at room temperature, and the platelet-rich plasma was pipetted off and placed into plastic centrifuge tubes. The platelet-rich plasma was then centrifuged at 1000 × *g* for 15 min at room temperature, and the platelet pellet was resuspended in buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM dextrose, and 10 μM indomethacin at a final pH 7.4) to a concentration of 2.5 × 10^8 platelets/ml. $CaCl_2$ was added to a final concentration of 500 μM. Platelet aggregation was carried out in a Chronolog model 300 aggregometer (Havertown, PA) using previously published methods (12). The washed platelets (425 μl) were added to individual silanized glass cuvettes and preincubated with 25 μl of epinephrine (10 μM) and 25 μl of antagonist ^{127}I-PTA-OH (Fig. 1) or vehicle for 1 min at 37°. This was followed by the addition of the aggregatory agent (25 μl), the stable endoperoxide analog U46619 (5) (final concentration of 20 nM to 50 μM), and the aggregation response was observed for 2 min. The percent aggregation response was determined at 1 min following the addition of U46619. Canine platelets have been previously shown to be unresponsive to TXA_2 mimetics or arachidonic acid (10, 11), but this unresponsiveness is reversed with the



n=1	R=H	ONO-11120
n=1	R=4-OH	PTA-OH
n=1	R=4-OMe	PTA-OM
n=1	R=3-Iodo,4-OH	I-PTA-OH
n=0	R=H	PTA-(ω-1)

FIG. 1. *Composite structure for the antagonists used in these studies*
All five are analogs of ONO-11120 and consist of an equal mixture of *R* and *S* configurations about the 15-hydroxy position.

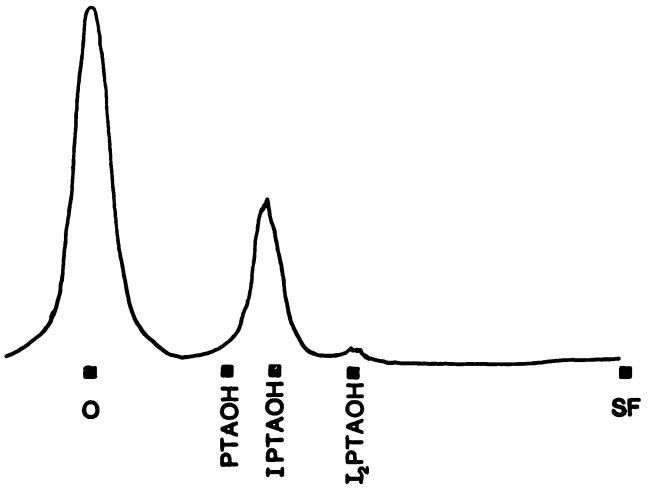


FIG. 2. *Tracing of a representative radiochromatogram of a TLC plate used to separate reaction products after radioiodination*
SF is the solvent front.

addition of subaggregatory concentrations of epinephrine. Therefore, epinephrine was included in the washed platelets to facilitate aggregation induced by U46619. The pA_2 value for ^{127}I-PTA-OH was determined by constructing dose-response curves of U46619 in the presence and absence of varied concentrations of ^{127}I-PTA-OH (13, 14).

Binding of ^{125}I-PTA-OH to washed platelets. Incubations (200 μl) containing 5×10^7 platelets were performed in silanized glass tubes (12 × 75 mm) at 37° for 30 min. The incubation media consisted of the Tris-NaCl buffer described above and ∿0.2 nM (∼10^5 cpm) ^{125}I-PTA-OH/tube. For equilibrium binding and inhibition experiments, various concentrations of agonists or antagonists ranging from 10^{-9} to 10^{-4} M were also included. The incubation was initiated by adding 100 μl of the washed platelet preparation and terminated by the addition of 4 ml of ice-cold 50 mM Tris, 100 mM NaCl buffer at pH 7.4, followed by rapid filtration through Whatman GF/C glass fiber filters. The filters were washed three more times with 4 ml of ice-cold buffer. The filtration procedure was complete within 10 sec. Noninhibitable binding was defined initially as that binding remaining in the presence of 100 μM ^{127}I-PTA-OH using previously described methods (15). Subsequently, 25 μM ^{127}I-PTA-OH was used regularly rather than 100 μM. Inhibitable binding typically was 60% of the total binding, and binding of ^{125}I-PTA-OH to the filters was approximately 0.6% of the total added cpm. Experiments for determining the association rate were carried out by incubating platelets for varying lengths of time in the presence of ^{125}I-PTA-OH (∿10^5 cpm) and 10 nM ^{127}I-PTA-OH. Termination of the reaction was carried out as described above. For dissociation experiments, a single incubation of 10 ml of washed platelets was carried out in the presence of ^{125}I-PTA-OH (∿0.2 nM). The reaction mixture was incubated for 30 min to allow equilibrium to be achieved, and an aliquot (200 μl) was removed and terminated as described above. This defined total binding (B_0) at the zero time point. At this time, ^{127}I-PTA-OH was added, yielding a final concentration of 25 μM. At selected times thereafter, aliquots (200 μl) were removed and terminated as described above. In experiments testing the effect of ADP and epinephrine on binding, ADP (5 μM) or epinephrine (10 μM) were incorporated into the incubation media prior to the addition of the platelet preparation. Ca^{2+} was excluded from the media during these experiments to prevent aggregation. In this manner, the platelets were exposed to these agents for the same amount of time while equilibrium was achieved.

Statistics. The equilibrium binding data were corrected for nonspecific binding and fitted to a 4-parameter model identifying two independent binding sites using an iterative simplex computer program for nonlinear regression analysis (16, 17). Alternatively, the data were not corrected for nonspecific binding, but were fitted to a 6-parameter model identifying three independent binding sites.

RESULTS

Determination of the pA_2 value for I-PTA-OH. To determine if ^{127}I-PTA-OH is a competitive antagonist of the platelet TXA_2/PGH_2 receptor in washed canine platelets, platelets were aggregated with varying concentrations of U46619, a TXA_2/PGH_2 receptor agonist (18), in the presence of epinephrine (10 μM). Dose-response curves were constructed for U46619, and the EC_{50} values (40 ± 5 nM, $n = 3$) were obtained directly from the curve. Dose ratios were calculated by comparing EC_{50} values in the presence of ^{127}I-PTA-OH to those in the presence of its vehicle. The pA_2 value obtained from the Schild plot was 7.97 (Fig. 3). The slope of the regression line was not significantly different from −1 (−0.95 ± 0.05, $n = 3$), indicating that ^{127}I-PTA-OH appears to be a competitive inhibitor of U46619-induced aggregation of washed canine platelets. The pharmacologic K_d (11 nM) was determined from the pA_2 value.

Binding of ^{125}I-PTA-OH to washed platelets. Having established that ^{127}I-PTA-OH was a competitive TXA_2/PGH_2 receptor antagonist, binding of ^{125}I-PTA-OH to washed canine platelets was characterized. The effect of pH on binding was examined, and a maximum value in inhibitable counts bound was obtained between pH 7.0 and 7.4 with a rapid fall in the binding occurring on either side of this range (Fig. 4). Inhibitable binding was linear over a platelet concentration range of 1×10^8 to 5×10^9/ml. Total and inhibitable binding were not affected by extracellular Ca^{2+} or Mg^{2+} in concentrations up to 750 μM (data not shown). All subsequent binding experiments were carried out at pH 7.4, a platelet concentration of 2.5×10^8/ml, and a final concentration of 500 μM $CaCl_2$. This buffer medium duplicated the conditions used in the aggregation studies.

The association rate constant (k_1) for the ligand-bind-

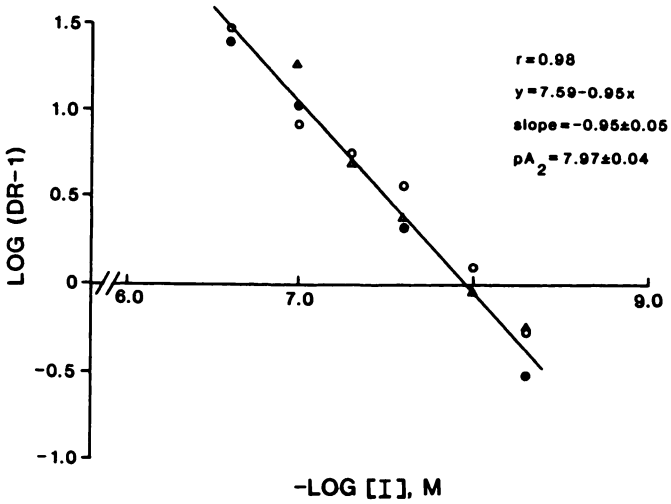


FIG. 3. *Schild plot for ^{127}I-PTA-OH in washed canine platelets*
○, ●, and △ represent three individual experiments. The slope obtained by linear regression analysis was not significantly different from −1. The K_d was estimated to be 11 nM.

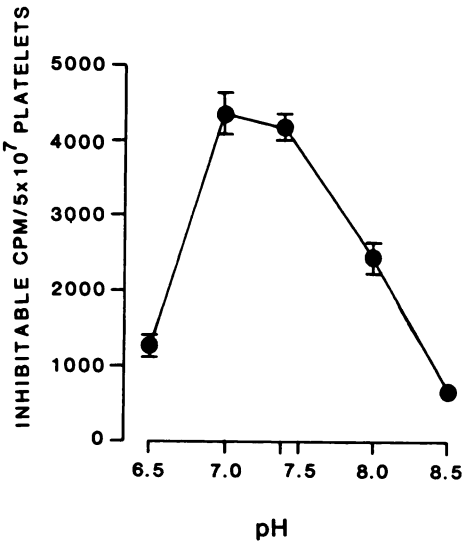


FIG. 4. *The effect of pH on specific binding of ^{125}I-PTA-OH*
Nonspecific binding was defined as the amount of ^{125}I-PTA-OH bound in the presence of 25 μM ^{127}I-PTA-OH. The values are the mean ± SEM ($n = 3$).

ing site complex was determined from the time course of binding of ^{125}I-PTA-OH plus ^{127}I-PTA-OH (10 nM). Binding appeared to approach steady state within 15 min at 30° (Fig. 5). The increase in binding with time is presented as a pseudo first-order rate plot (Fig. 5, *inset*). The observed rate constant (k_{obs}) is given by the slope and was found to be 1.90×10^{-1} min^{-1} (standard deviation of the slope was ±0.08). To determine the rate constant (k_{-1}) for the dissociation of the ligand-binding site complex, washed platelets were incubated with ^{125}I-PTA-OH (10 nM) for 30 min at 30° to ensure that equilibrium had been reached. At an arbitrary zero time point, ^{127}I-PTA-OH was added to a final concentration of 25 μM, and the decrease in binding of the radioligand was determined at various times. An exponential decrease in binding was observed (Fig. 6) from which the data could be replotted to give a line, the negative slope of which is a measure of the dissociation (k_{-1}) rate constant (Fig. 6, *inset*). The value for k_{-1} was found to be 1.45×10^{-1} min^{-1} (standard deviation of slope was ±0.06). The value of k_1 for the association of the ligand-receptor complex is determined by: $k_1 = (k_{obs} - k_{-1})/[L]$, where $[L]$ is the ligand concentration. The calculated value of k_1 was found to be 4.50×10^6 M^{-1} min^{-1}. Since $K_d = k_{-1}/k_1$, the kinetically determined K_d value was 32 nM.

The binding of a fixed concentration of ^{125}I-PTA-OH (~0.2 nM) to washed platelets was measured at steady state with increasing concentrations of ^{127}I-PTA-OH up to 25 μM (Fig. 7). Scatchard analysis of the data revealed a nonlinear plot, consistent with two independent binding sites, one of high and one of low affinity or negative cooperativity (19). Computer analysis revealed a K_d of 24 nM for the high affinity site (Fig. 7, *inset*) and 2.1 μM for the low affinity site. The maximum binding capacity of the high affinity site was 71 fmol/10^7 platelets (1.36 pmol/mg protein). This yields 4400 high affinity binding sites/platelet.

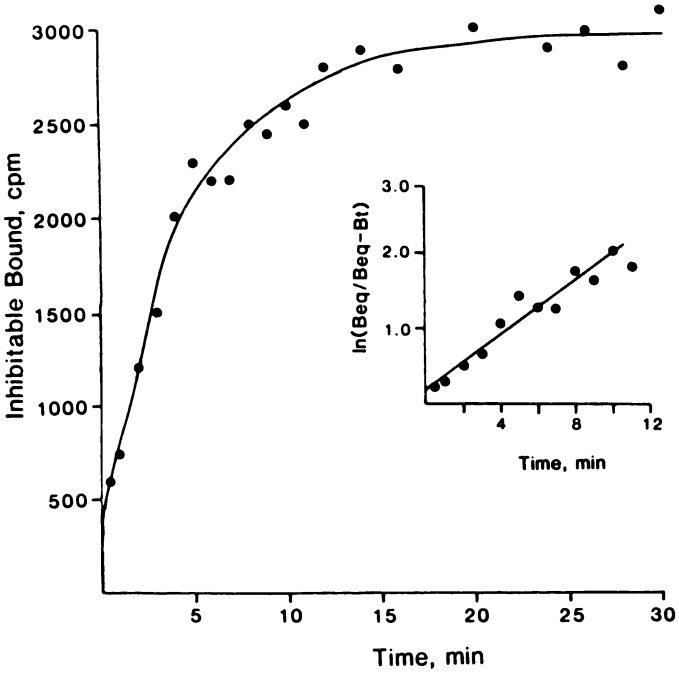


FIG. 5. *The association of ^{125}I-PTA-OH + ^{127}I-PTA-OH (10 nM) to washed canine platelets*

Each data point represents the mean of three separate experiments. Within each experiment, the points represent duplicate determinations. The *inset* shows the pseudo first-order rate plot of the same data, where B_{eq} is the specific binding at equilibrium and B_t is the specific binding at any particular time t.

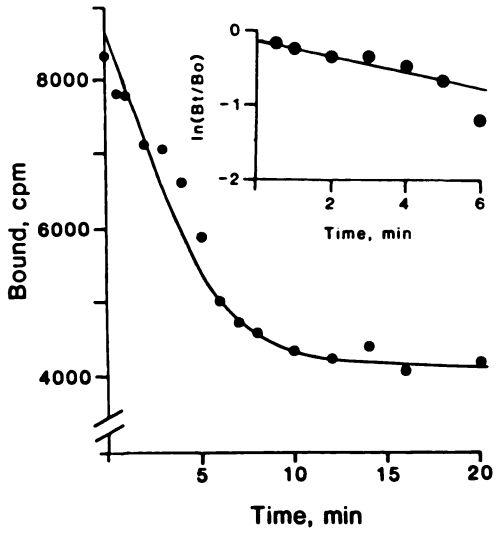


FIG. 6. *Dissociation of ^{125}I-PTA-OH from washed canine platelets*

At time = 0, ^{127}I-PTA-OH was added for a final concentration of 25 μM. The plot shows the dissociation of the ligand-receptor complex with respect to time. Each data point represents the mean of two experiments with duplicate determinations within each experiment. The *inset* shows the logarithmic replot of the data corrected for non-inhibitable binding where B_t is the ^{125}I-PTA-OH specifically bound at any particular time t and B_0 is the amount specifically bound at time zero.

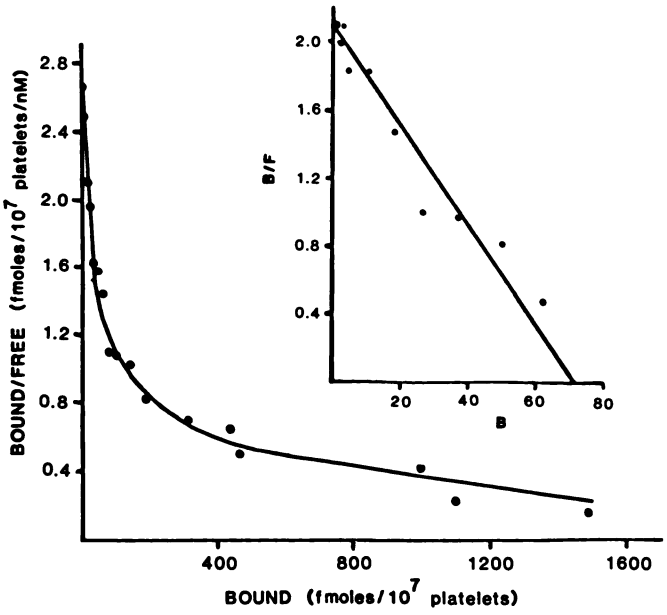


FIG. 7. *Scatchard plot for the binding of I-PTA-OH to washed platelets*

The *inset* shows the Scatchard plot of the high affinity site using a computer analysis ($r = 0.97$). The data presented are the result of five separate experiments and are corrected for noninhibitable binding.

Inhibition of ^{125}I-PTA-OH binding by antagonists. A number of compounds were examined for their ability to inhibit ^{125}I-PTA-OH (0.2 nM) from washed platelets. The structures of the antagonists are shown in Fig. 1 and the inhibition curves in Fig. 8. ONO-11120, PTA-OM, PTA-OH, and PTA-(ω-1) had IC_{50} values of 14 ± 8, 25 ± 10, 50 ± 6, and 150 ± 52 nM, respectively. The IC_{50} values for the antagonists for inhibition of ^{125}I-PTA-OH from its binding site correlate significantly ($r = 0.97, p < 0.01$) with their potencies as antagonists of U46619-induced platelet aggregation in canine platelet-rich plasma (9). $PGF_{2\alpha}$ and PGE_2 inhibited the radiolabeled ligand binding but only at much greater concentrations, yielding IC_{50} values of 7.0 and 8.1 μM, respectively.

Effect of ADP and epinephrine on binding. Since it had been shown that canine platelets do not aggregate to arachidonic acid or TXA_2 mimetics except in the presence of subthreshold concentrations of epinephrine (10), we determined the effect of epinephrine on the binding characteristics of ^{125}I-PTA-OH. In addition, the effects of ADP were examined since it has been shown to increase the number of fibrinogen receptors in human platelets (20). As can be seen in Table 1, neither ADP (5 μM) nor epinephrine (10 μM) had an effect upon the K_d or B_{max} of either the high or low affinity binding sites for ^{125}I-PTA-OH. Since changes in binding characteristics may not necessarily be reflected when inhibitions are done with an antagonist, but become apparent with an agonist (21), the ability of U46619 to displace ^{125}I-PTA-OH from washed platelets was also examined. The inhibition curves for U46619 both in the absence and presence of epinephrine (10 μM) are shown in Fig. 9. The inhibition curves yielded essentially an identical profile with IC_{50} values of 38 and 43 nM in the absence and presence of epinephrine, respectively.

DISCUSSION

Until recently, the characterization of TXA_2/PGH_2 receptors has utilized predominantly pharmacologic approaches. The use of radiolabeled ligands for characterization of the receptors has lagged behind the pharmacologic approach. The major reason for this has been the unavailability of a suitable radiolabeled ligand. In particular, efforts have been hampered by the lack of molecules with high affinity for the receptor which may be easily radiolabeled to high specific activity and yet retain biological activity. Previous binding studies of TXA_2/PGH_2 receptors have utilized ^{3}H-labeled compounds (22, 23) as the ligand in both washed platelets and platelet membranes. More recently, we have described the potential for using an ^{125}I-labeled ligand in such studies (24). That report described the potential for making changes in the bottom (ω) side chain of a synthetic TXA_2/PGH_2 antagonist which would allow easy introduction of ^{125}I. However, lack of potency (K_d in low μM range) for this antagonist hindered its use for extensive investigations of platelet TXA_2/PGH_2 receptors. Therefore, a series of compounds was synthesized that were derivatives of 13-azapinane-TXA_2, a more potent series of TXA_2/PGH_2 receptor antagonists (8). One of these compounds was PTA-OH which possessed the necessary functionality (phenol ring) to be iodinated to yield I-PTA-OH (Fig. 1). The pharmacology of these compounds in platelets and blood vessels indicated that this series of compounds, in particular I-PTA-OH, may be suitable for binding studies when radiolabeled (9). This study demonstrates that I-PTA-OH retains its biological activity as a TXA_2/PGH_2 receptor antagonist and that ^{125}I-PTA-OH can be used to study these putative receptors.

The pharmacologically determined K_d for ^{127}I-PTA-OH obtained from dose-response curves with the TXA_2/PGH_2 mimetic U46619 gave a K_d of 11 nM. In addition, the slope was not significantly different from −1, a necessary criterion for a competitive antagonist. These results suggest that ^{127}I-PTA-OH acts in a competitive fashion in antagonizing U46619-induced platelet aggregation. The kinetically determined K_d, as calculated by k_{-1}/k_1, was 32 nM. From steady state binding experi-

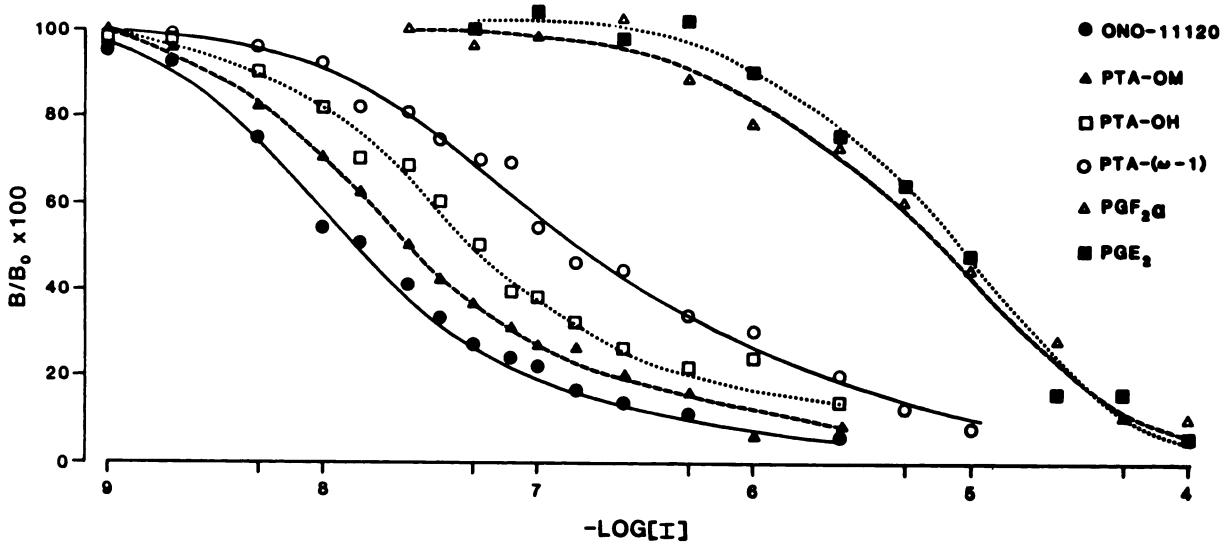


FIG. 8. *Inhibition of ^{125}I-PTA-OH binding from washed canine platelets by ONO-11120, PTA-OM, PTA-OH, PTA-(ω-1), PGE_2, and $PGF_{2\alpha}$.* $n = 3$ per antagonist; $n = 2$ for PGE_2 and $PGF_{2\alpha}$.

TABLE 1

Effect of ADP and epinephrine on the binding characteristics of ^{125}I-PTA-OH to washed canine platelets

Data are expressed as $\bar{X}$ ± S.E.M. Values represent three separate determinations. No significant differences were obtained between any of the groups. K_h is the K_d for the high affinity site and K_L is the K_d for the low affinity site. B_h is the B_{max} for the high affinity site, and B_L is the B_{max} for the low affinity site.

	K_d		B_{max}	
	K_h	K_L	B_h	B_L
	nM		*fmoles/10^7 platelets*	
Control	27 ± 10	2330 ± 720	64 ± 12	1510 ± 384
ADP (5 μM)	28 ± 5	1990 ± 750	58 ± 8	1410 ± 500
Epinephrine (10 μM)	33 ± 4	3230 ± 1020	72 ± 12	1872 ± 650

ments, a high affinity site with a K_d of 24 nM and a low affinity site of 2.1 μM were found. The K_d of the high affinity site agrees with the K_d values obtained pharmacologically and kinetically, suggesting that this high affinity binding site is the site responsible for inhibition of aggregation induced by U46619. No function for the low affinity binding site has been identified. However, it may represent nonspecific binding in the platelet, binding to an amine transport process, or binding to a metabolic enzyme. With regard to the latter possibility, binding of ^{125}I-PTA-OH to a metabolic enzyme could result in metabolism of ^{125}I-PTA-OH. In experiments where ^{125}I-PTA-OH was incubated with platelets followed by extraction and thin layer chromatography, all the radioactivity was recovered in the spot corresponding to I-PTA-OH, suggesting that there was no significant metabolism of ^{125}I-PTA-OH by the platelets (data not show).

In previous studies, we established the rank order for a series of 13-azapinane-TXA_2/PGH_2 antagonists to inhibit U46619-induced platelet aggregation in canine platelet-rich plasma (9). That study has been extended here by showing that this series of antagonists displaced ^{125}I-PTA-OH from its binding site in washed canine platelets. These 13-azapinane derivatives of TXA_2 all displaced ^{125}I-PTA-OH with IC_{50} values in the low nanomolar range. In addition, these compounds exhibited a rank order which was not significantly different from that observed for their ability to antagonize U46619-induced canine platelet aggregation in platelet-rich plasma, suggesting that ^{125}I-PTA-OH is binding to a pharmacological receptor (9). $PGF_{2\alpha}$ and PGE_2 also displaced the radioligand but only at concentrations that ranged from 100 to 1000 times that required for the 13-azapinane-TXA_2 analogs. With IC_{50} values of nearly 10 μM, both PGE_2 and $PGF_{2\alpha}$ may be cross-reacting with the putative TXA_2/PGH_2 receptor rather than binding specifically to the TXA_2/PGH_2 receptor or a common prostanoid receptor as pointed out previously (25).

Previous studies have shown that the response of platelets to TXA_2/PGH_2 mimetics and antagonists appears to be species-dependent (11, 26). For example, studies have indicated that a majority of dogs have platelets which are unresponsive to TXA_2/PGH_2 mimetics or arachidonic acid (10). This unresponsiveness could be reversed by preincubating the platelets with subaggregating concentrations of epinephrine. This reversal in responsiveness to TXA_2 mimetics and arachidonic acid was suggested to be coupled to decreased intraplatelet cAMP levels (10). Alternatively, since U46619-induced canine platelet aggregation could be blocked in a dose-dependent manner by the antagonists used in this study (9), epinephrine may have unmasked additional TXA_2/PGH_2 receptors in addition to or instead of altering endogenous cAMP levels. Furthermore, it is known that fibrinogen receptors, not normally exposed at the surface of platelets, are up-regulated in the presence of epinephrine and ADP (20). Neither ADP nor epinephrine induced a significant change in the K_d or B_{max} TXA_2/PGH_2 values of ^{125}I-PTA-OH binding either to the high or low affinity binding site. Since changes in the K_d may not be apparent when using a radiolabeled antagonist but can be revealed by competition with an agonist (21), binding studies with U46619 in the absence (control) or presence

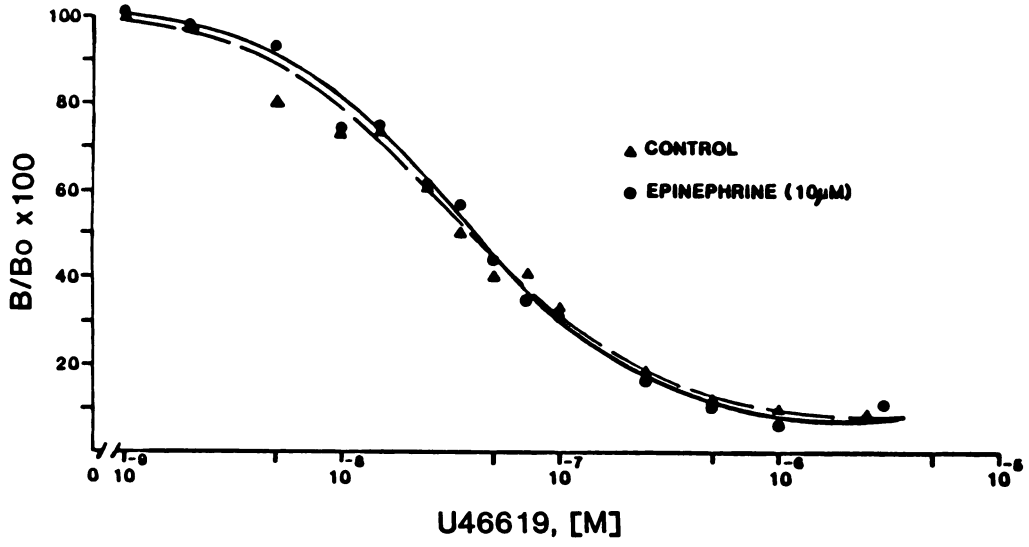


FIG. 9. *Inhibition of ^{125}I-PTA-OH binding from washed canine platelets by U46619 in the absence (▲) and presence (●) of 10 μM epinephrine (n = 2)*

of epinephrine (10 μM) were also examined. Epinephrine did not produce a significant qualitative or quantitative change in the inhibition curve. These results suggest that ADP and epinephrine are not altering the density or affinity of TXA_2/PGH_2 receptors and may act at a post-receptor site, possibly adenylate cyclase. This conclusion agrees with the previous reports suggesting that this unresponsiveness was coupled to elevated cAMP levels in the platelet and that the role of epinephrine was to decrease these cAMP levels. While we have not confirmed this directly, the evidence presented here suggests that a change in the TXA_2/PGH_2 receptor (affinity or number) is not affected by such agents as epinephrine or ADP, agents which are known to affect other receptors in platelets (20).

In summary, we have described the binding characteristics of a TXA_2/PGH_2 receptor antagonist, ^{125}I-PTA-OH, to washed canine platelets. Three independent measures of the affinity of this compound are reported and agree well. This agreement is observed in the high affinity site which appears to be the site responsible for platelet aggregation. The rank order potency for a series of antagonists to displace ^{125}I-PTA-OH agrees very well with their rank order for antagonism of U46619-induced aggregation in canine PRP. Thus, evidence exists for the presence of a TXA_2/PGH_2 receptor in dog platelets.

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Send reprint requests to: Dr. Perry V. Halushka, M.D., Ph.D., Professor of Pharmacology and Medicine, Department of Pharmacology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425.