

Distinct platelet thromboxane A₂/prostaglandin H₂ receptor subtypes. A radioligand binding study of human platelets.

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Research Article

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Distinct Platelet Thromboxane A₂/Prostaglandin H₂ Receptor Subtypes

A Radioligand Binding Study of Human Platelets

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Abstract

Thromboxane A₂ (TXA₂) and prostaglandin H₂ (PGH₂) may aggregate platelets via a common membrane receptor(s). To further characterize this receptor, binding of the radiolabeled TXA₂/PGH₂ mimetic [¹²⁵I]BOP to washed human platelets (WP) was investigated. [¹²⁵I]BOP was competitively displaced from its platelet binding site by stable TXA₂/PGH₂ analogues. Competition curves were shallow with Hill coefficients of -0.73 ± 0.05 ($P < 0.001$ different from unity) ($90 \pm 1\%$ specific binding). Scatchard plots were curvilinear and most consistent with two binding sites; a high-affinity site with K_d of 234 ± 103 pM, B_{max} of 0.7 ± 0.3 pM/mg protein (180 ± 87 sites/WP), and a lower affinity site with K_d of 2.31 ± 0.86 nM, B_{max} of 2.2 ± 0.3 pM/mg protein (666 ± 65 sites/WP). [¹²⁵I]BOP association and dissociation kinetics gave a K_d of 157 pM without evidence of negative cooperativity. The EC₅₀ for I-BOP-induced initial Ca²⁺ increase was 209 ± 24 pM, shape change was 263 ± 65 pM, and aggregation was 4.4 ± 0.5 nM.

Parallel binding studies using the TXA₂/PGH₂ receptor antagonist [¹²⁵I]PTA-OH showed a single binding site. The rank order for TXA₂/PGH₂ analogues to displace [¹²⁵I]PTA-OH was identical to that for [¹²⁵I]BOP.

These studies indicate that [¹²⁵I]BOP binds to two distinct sites on human platelets that may represent platelet TXA₂/PGH₂ receptor subtypes. The close correlation of IC₅₀ values for I-BOP-induced platelet shape change and aggregation with the two K_d s for [¹²⁵I]BOP binding suggests that these platelet responses may be independently mediated by the two putative receptors.

Introduction

Thromboxane A₂ (TXA₂) and prostaglandin H₂ (PGH₂) are synthesized from AA and released by platelets in response to a

variety of stimuli. These autocooids result in platelet shape change/aggregation and vascular smooth muscle contraction through interaction with specific membrane receptors (1). Due to the indistinguishable effects of these agents upon platelets, a common notation has been to label their combined putative receptor as TXA₂/PGH₂ (2). However, some studies have suggested that separate platelet receptors exist for the endoperoxide PGH₂ and TXA₂ (3, 4). The thromboxane receptor has been postulated to mediate platelet shape change and the endoperoxide receptor has been postulated to mediate aggregation (3). At present, definitive studies demonstrating distinct subtypes of TXA₂/PGH₂ receptors are lacking.

The short half-life of TXA₂ (~ 90 s) and PGH₂ (~ 5 min) at physiologic pH (5) precludes direct studies of the binding of these substances to their putative receptor(s). However, stable endoperoxide and thromboxane analogues have been developed, radiolabeled, and successfully used in equilibrium binding studies to the human platelet TXA₂/PGH₂ receptor.

The stable endoperoxide mimetic [³H]U46619¹ has been used to bind to human platelets but interpretation of the results has been complicated by markedly varying dissociation constants (K_d) and binding capacities (6–8). It is possible that U46619 lacks sufficient potency and/or specific activity to be a useful ligand for investigation of TXA₂/PGH₂ receptor subtypes.

Detailed studies of the human platelet TXA₂/PGH₂ receptor have also been performed with [¹²⁵I]PTA-OH, a stable radioiodinated TXA₂ antagonist. Two different groups have reported that [¹²⁵I]PTA-OH binds to a single site with a capacity of ~ 2,000 sites per platelet (9, 10). Though [¹²⁵I]PTA-OH has a higher specific activity and greater affinity for the receptor than does [³H]U46619, its properties as an antagonist may make it less than ideal for discriminating between different TXA₂/PGH₂ agonist binding sites (11–13).

Because of the divergent results regarding the affinity and density of TXA₂/PGH₂ receptors in prior studies of human platelets with [³H]U46619 and [¹²⁵I]PTA-OH, this study used a novel radioiodinated TXA₂ mimetic ligand, [¹²⁵I]BOP, the most potent radiolabeled TXA₂/PGH₂ agonist yet described (14). [¹²⁵I]BOP was used in binding studies to washed human platelets to further investigate the possibility that human platelets possess distinct subtypes of TXA₂/PGH₂ receptors that may represent separate endoperoxide and thromboxane receptors.

Methods

Subjects. Blood (40 ml) was obtained by antecubital venipuncture through nineteen gauge needles into syringes containing indomethacin (10 μM) and EDTA (5 mM) (final concentrations) from six normal healthy volunteers who had not taken any medication for the previous week. Each subject gave written informed consent. This study was

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1. Abbreviations used in this paper: I-BOP, [15-(1 α ,2 β (5Z),3 α -(1E,3S),4 α)]-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl)-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; I-PTA-OH, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15 α β - ω -tetranor-TXA₂; SQ29,548, [[1S]1 α ,2 β (5Z),3 β ,4 α]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy 11 α ,9 α (epoxymethano) prosta-5Z,13E-dienoic acid.

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approved by the University of Texas Health Science Center at San Antonio Institutional Review Board.

Materials. Fig. 1 shows the structures of the TXA₂/PGH₂ analogues used in these studies. The optically active amine precursor of I-BOP and [¹²⁵I]BOP, PTA-OH, [¹²⁵I]PTA-OH, U46619, and SQ29,548 were gifts from Dr. Dale Mais and Dr. Perry Halushka, Department of Cell and Molecular Biology and Experimental Therapeutics, Medical University of South Carolina, Charleston, South Carolina. Stock solutions of these compounds were maintained in absolute ethanol. Working solutions, prepared by evaporating stock solutions to dryness under nitrogen gas and dissolving them in vehicle (0.038 M NaOH in distilled water), were stored at -20°C. Aequorin was purchased from Dr. John Blinks, Department of Pharmacology, Mayo Clinic, Rochester, Minnesota. [¹²⁵I]Na was obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest purity available from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX).

Radioiodination of ligands. The initial iodination procedure for I-BOP and I-PTA-OH is identical and follows closely the method of Mais (9). Briefly, 10 μl of stock ethanolic solution (1.0 M) of the precursor compound was diluted into 40 μl of 0.1 M phosphate buffer, pH 7.5 in a siliconized borosilicate glass test tube. To this solution the following were added in order: 1.5 mCi [¹²⁵I]Na (15 μl) and 5 μl chloramine T (0.3 mg/ml). The reaction was allowed to proceed for 45 s with gentle mixing at room temperature after which another 5 μl of chloramine T was added. After an additional 45 seconds the reaction was stopped by the addition of 30 μl of sodium metabisulfite (50 mg/ml).

¹²⁵I-labeled product was purified on a dual pump HPLC system (Beckman Instruments, Fullerton, CA) using a Partisil-5-ODS-3 reverse phase column (Whatman Instruments, Clifton, NJ) and eluted with a mobile phase of methanol-0.1 M ammonium acetate (60:40) at a flow rate of 1 ml/min. Under these conditions [¹²⁵I]PTA-OH eluted at 10 min and [¹²⁵I]BOP-amine eluted at 11.6 min. [¹²⁵I]PTA-OH was diluted in methanol and stored at -20°C.

[¹²⁵I]BOP was prepared by deamination of the radioiodinated amine as follows. The HPLC fractions were evaporated to dryness under nitrogen and dissolved in 50 μl of absolute ethanol to which 5 μl of glacial acetic acid and 10 μl of NaNO₂ (100 mg/ml) was added during vortex agitation. The reaction proceeded at room temperature for 20 min and was diluted to 100 μl total volume with distilled water. HPLC of the product using the system described above and a mobile phase of methanol-0.1 M ammonium acetate (63:37) at 1 ml/min resulted in elution of [¹²⁵I]BOP at 15 min. The HPLC fractions containing peak levels of radioactivity were pooled and stored at -20°C until use.

Radiolabeled ligands were prepared the day of use by evaporation under nitrogen gas at room temperature and dissolving in the appropriate volume of assay buffer (25 mM Hepes, 150 mM NaCl, 10 μM indomethacin, pH 7.4).

Platelet aggregation and [Ca]_i. For aggregation studies washed platelets were prepared by differential centrifugation and resuspended in assay buffer to a final concentration of 2.5 × 10⁸ platelets/ml. Ca²⁺ was adjusted to a final concentration of 1 mM. Platelet aggregation was carried out in a platelet ionized calcium aggregometer (model 600; Chrono-Log Corp., Havertown, PA). Washed platelets (475 μl) were added to siliconized glass cuvettes and incubated for 1 min at 37°C before the addition of agonist (25 μl). The aggregation response was observed for 4 min and dose-response curves were constructed. The EC₅₀ value, defined as the concentration of agonist that produced a half-maximal aggregation response at 3 min, was determined from log-logit transformation of the concentration-response curves.

For measurement of [Ca]_i, platelets were loaded with the photo-protein aequorin using the method of Yamaguchi (15). Aequorin was chosen for measurement of [Ca]_i because of its sensitivity to calcium transients and because it was possible to simultaneously quantify the platelet aggregation/shape change response and change in [Ca]_i using a lumiaggregometer. Briefly, the platelet pellet from 40 ml of blood was suspended in 90 μl Hepes-buffered saline (140 mM NaCl, 2.7 mM KCl, 0.1% bovine albumin, 0.1% dextrose, 3.8 mM Hepes, pH 7.6)

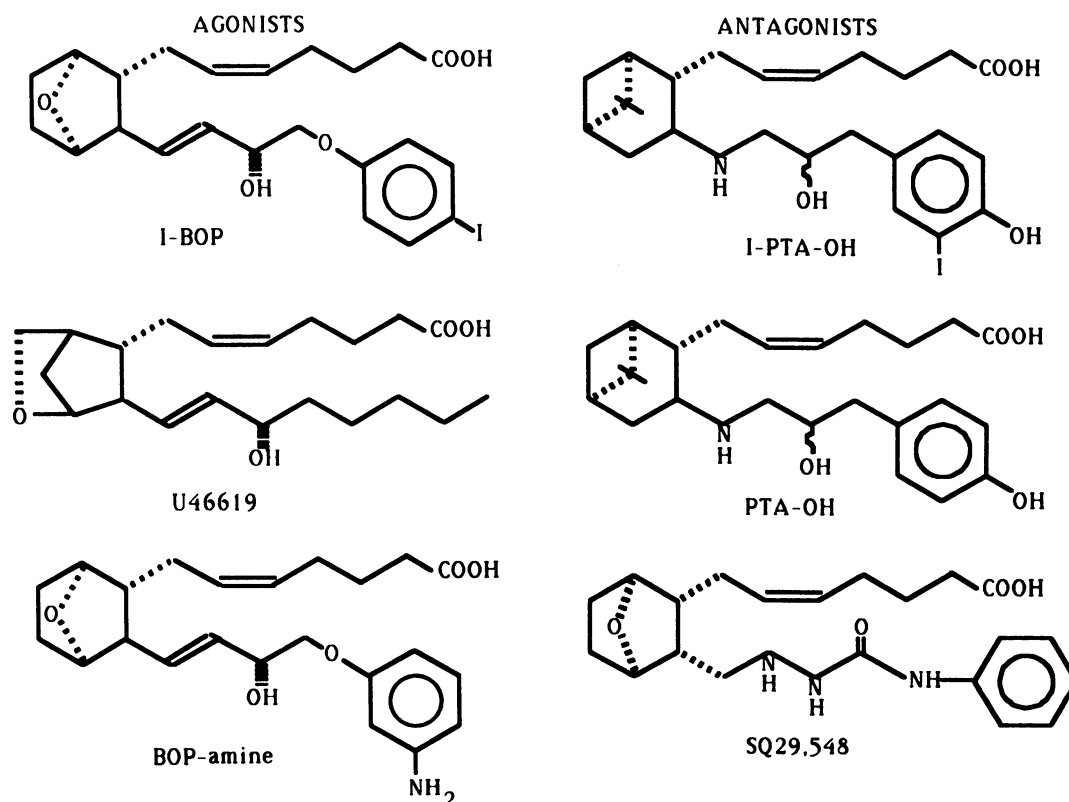


Figure 1. Chemical structures of the three TXA₂/PGH₂ receptor agonists and the three antagonists used in these studies.

with 5 mM EDTA (final concentration of platelets $\sim 10^9/\mu\text{l}$) to which 10 μl of aequorin (3 mg/ml) was added. DMSO (1 μl) was added at 1.5-min intervals for 7.5 min to a final concentration of 6%. After incubation for 2 min, the platelets were diluted with 10 ml of Hepes-buffered saline and centrifuged at 10,000 g for 90 s to obtain a platelet pellet. The platelets were washed once in an identical manner in Hepes-buffered saline and finally suspended in Hepes-buffered saline plus 1 mM Ca^{2+} , 1.25 mM Mg^{2+} (2.5×10^8 platelets/ml). Aequorin luminescence of 1 ml of washed platelets in plastic cuvettes was monitored in the lumiaggregometer and aggregation/shape change and luminescence were recorded simultaneously on a dual-channel recorder (Chrono-Log Corp.). The aequorin luminescence signal was recorded directly or, with an amplifier/integrator (Chrono-Log Corp.), as the integrated area of the luminescence curve. All calculations were performed using the signal integral measured in this manner.

Platelet binding of [^{125}I]PTA-OH. [^{125}I]PTA-OH binding to washed platelets was determined using a modification of the method of Mais (9). Briefly, washed platelets (100 μl) diluted to a concentration of $5 \times 10^8/\text{ml}$ in the same assay buffer used in the aggregation experiments were incubated in the presence of competing ligand (0.01 nM to 1 μM final concentration) in a total volume of 200 μl for 30 min at 37°C in a shaking water bath with [^{125}I]PTA-OH (70,000 cpm). [^{127}I]PTA-OH (1 μM) was added to determine displaceable binding which was $60 \pm 1\%$ ($n = 6$). The assays were terminated by the addition of 4 ml of ice cold assay buffer and immediate vacuum filtration through GF/C glass fiber filters (Whatman Instruments) followed by three additional 4-ml washes with ice-cold assay buffer.

Platelet binding of [^{125}I]BOP. Binding of [^{125}I]BOP to washed platelets was assayed by a modification of the above method. Washed platelets (100 μl) were diluted to a concentration of $1 \times 10^8/\text{ml}$ in assay buffer. At this concentration of platelets the amount of bound ligand was typically 8–10% of the total added ligand. The platelets were incubated with [^{125}I]BOP (30,000 cpm) (total volume, 200 μl) at 37°C for 30 min in a shaking water bath. For equilibrium binding and displacement experiments, various concentrations (0.01 nM to 1 μM) of competing ligand were included. [^{127}I]BOP (1 μM) was added to determine displaceable binding which was $90 \pm 1\%$ ($n = 6$) of total binding. The reaction was terminated as described above.

Experiments to determine the time course of association and dissociation of [^{125}I]BOP to washed platelets were carried out at 20°C because association of the ligand at 37°C was essentially complete within 4 min at the lowest concentrations used and therefore could not be quantified with confidence (data not shown). Experiments to determine the association rate were performed by incubating platelets for various periods of time in the presence of [^{125}I]BOP (30,000–40,000 cpm) plus three concentrations of [^{127}I]BOP. Termination of the assay was carried out in the manner described above. The time course of dissociation was determined by incubating 5 ml of washed platelets with [^{125}I]BOP (0.03 nM) for 45 min at 20°C for association of the ligand to occur. Aliquots (200 μl) were then placed into individual tubes and dissociation was initiated either by addition of 1 μM [^{127}I]BOP or, in separate experiments, by dilution into 20 ml of assay buffer. The assay was terminated at various times by vacuum filtration as described above.

To determine if [^{125}I]BOP was somehow changed during incubation with platelets, the following experiment was performed. [^{125}I]BOP (final concentration of 200,000 cpm/100 μl ; 0.4 nM) plus 100 nM [^{127}I]BOP were incubated for 30 min under the usual binding experiment conditions with washed platelets ($5 \times 10^8/\text{ml}$). The platelets were pelleted by centrifugation and the supernatant, containing unbound I-BOP, was ultrafiltered through a 10,000-mol wt membrane (Amicon Corp., Danvers, MA) to remove contaminating proteins. A sample of the ultrafiltrate underwent HPLC in MeOH-0.1 M ammonium acetate (60:40) on the system described above.

Assay buffer plus [^{127}I]BOP (100 nM) and [^{125}I]BOP (0.4 nM) that had not been incubated with platelets was used as a standard. Under these conditions I-BOP (determined by absorbance at 254 nm and the peak of eluted radioactivity) eluted in identical narrow peaks at 10 min

in the samples which had and had not been incubated with platelets. Thus, incubation with platelets did not appear to alter I-BOP.

Protein content was determined by the Bradford method (16) using BSA as the reference standard.

Statistical methods. Displacement curves were analyzed by Hill plots with linear regression to determine the Hill coefficient (slope of the curve) and the IC_{50} (concentration of competing ligand required to displace 50% of the radiolabeled ligand from its binding site). A t test was used to determine if the Hill coefficients differed significantly from one and to compare IC_{50} values and Hill coefficients between ligands (17).

Displacement of [^{125}I]PTA-OH or [^{125}I]BOP by their [^{127}I] analogues was fitted to a nonlinear model using the LIGAND program (18), which provides statistical measures of goodness of fit, comparisons of one or multiple receptor models, and allows for simultaneous analysis of multiple curves with corrections for nonhomogeneity of variance. The following parameters were derived as least squares estimates: affinity of the binding site(s) for the ligand, capacity of binding for the ligand, and nonspecific binding. All experiments were initially fitted to a one-site model and subsequently to a two-site model. The two-site model was accepted over the single-site model only if it was a better fit as judged by a significant F statistic for simultaneous analysis of covariance of all of the curves. The specific activity of the radioiodinated ligands was assumed to be 2,200 Ci/mmol. The null hypothesis was rejected at $P < 0.05$. Data are reported as the mean \pm SEM.

Results

Platelet aggregation and $[\text{Ca}]_i$. Platelets aggregated to I-BOP in a dose-related fashion with an EC_{50} of 4.4 ± 0.4 nM ($n = 6$) (Fig. 2). Platelet shape change was still evident at concentrations of I-BOP that did not aggregate the platelets. The maximal shape change response was seen at concentrations of 1–2.5 nM. By amplifying the aggregometer signal 20-fold the shape change response was quantified and concentration-response curves were constructed. I-BOP induced shape change with an EC_{50} of 0.263 ± 0.065 nM ($n = 6$) (Fig. 2).

Aequorin-loaded platelets aggregated fully in response to I-BOP with no difference in concentration response (data not shown). The maximal $[\text{Ca}]_i$ induced by 100 nM I-BOP was 6.3 μM . The calcium signal at concentrations of I-BOP that resulted in high amplitude shape change but minimal aggregation (1–2.5 nM) was biphasic with the initial signal seen immediately after addition of the agonist followed by a second signal, which preceded aggregation by 2–3 s. Assuming that the initial signal was the most proximate response to I-BOP stimulation, a concentration-response relationship was obtained that closely paralleled that of shape change and that resulted in an EC_{50} of the initial phase $[\text{Ca}]_i$ of 0.209 ± 0.024 nM ($n = 3$) (Fig. 2).

Binding of [^{125}I]BOP to washed platelets. Binding of [^{125}I]BOP to washed platelets was of high affinity and saturable over [^{125}I]BOP concentrations of 0.005–10 nM (data not shown). $\text{TXA}_2/\text{PGH}_2$ agonist and antagonist competition curves for [^{125}I]BOP and [^{125}I]PTA-OH binding are shown in Figs. 3 and 4. [^{127}I]BOP competition of [^{125}I]BOP binding resulted in curves that were shallow with Hill coefficients of -0.73 ± 0.05 ($P < 0.001$ different from unity, $n = 6$). The mean concentration of [^{127}I]BOP that inhibited binding by 50% (IC_{50}) was 0.94 nM and the mean K_d determined by computerized nonlinear curve fitting (LIGAND) to a one-site model was 0.77 nM with a binding capacity of 2.5 pmol/mg protein (657 sites/platelet). The Scatchard plot of displaceable binding was nonlinear (Fig. 5, inset) indicating either multiple binding sites or negative

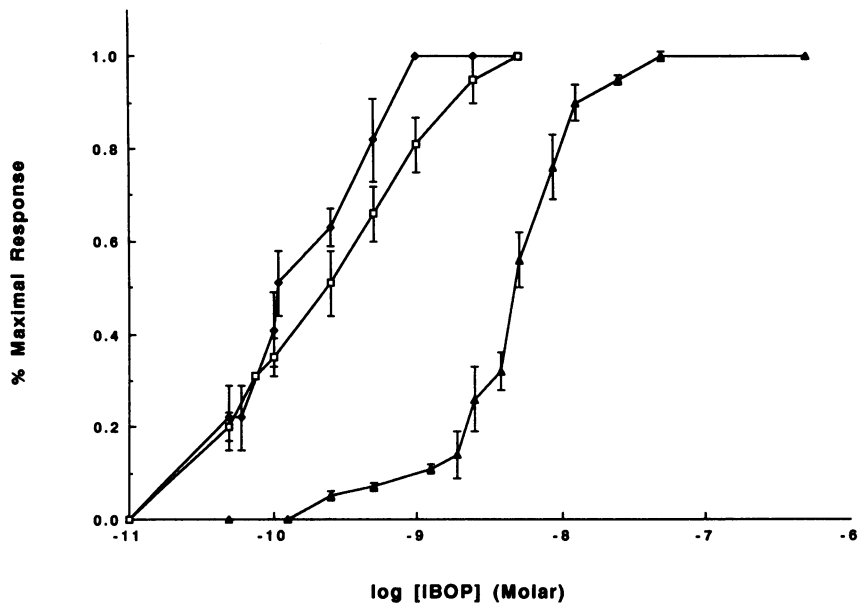


Figure 2. Concentration-response relationships for platelet aggregation (\blacktriangle), shape change (\square), and the initial phase of intracellular calcium rise ($[Ca]_i$) (\blacklozenge) induced by I-BOP. Each point represents the mean \pm SEM of six (aggregation and shape change) or three ($[Ca]_i$) separate experiments.

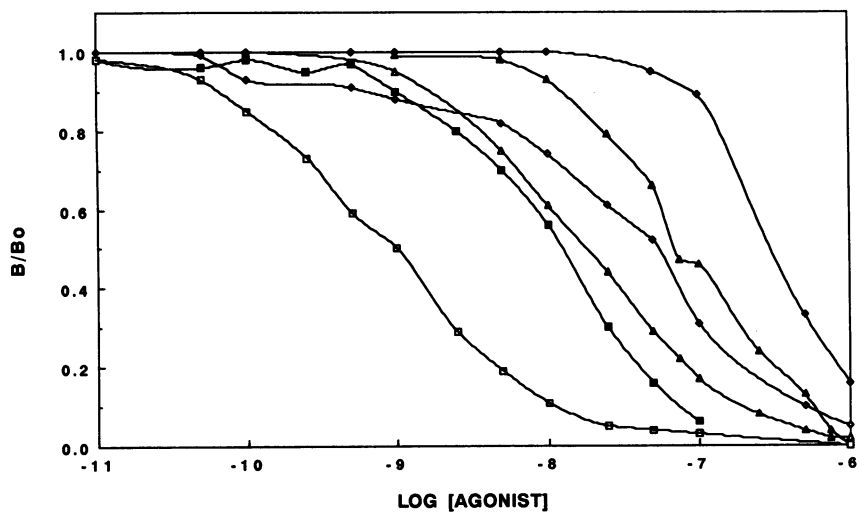


Figure 3. Displacement of the TXA₂/PGH₂ agonist ligand [¹²⁵I]BOP (open symbols) and the TXA₂/PGH₂ antagonist ligand [¹²⁵I]PTA-OH (solid symbols) by the TXA₂/PGH₂ mimetics [¹²⁷I]BOP (\blacksquare , \square), U46619 (\blacktriangle , \triangle), and BOP-amine (\blacklozenge , \lozenge). The data are presented as the means of duplicate determinations for four paired experiments per compound. SE bars were omitted for clarity.

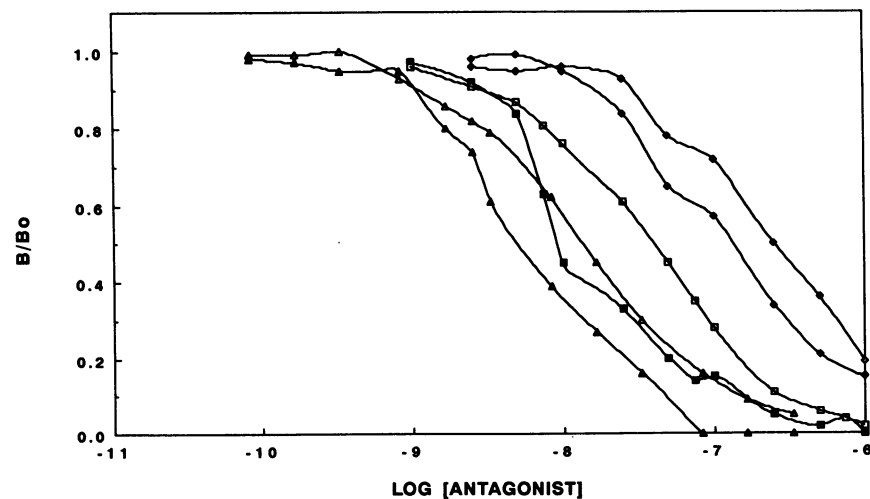


Figure 4. Displacement of [¹²⁵I]BOP (open symbols) and [¹²⁵I]PTA-OH (solid symbols) by the TXA₂/PGH₂ antagonists SQ29,548 (\blacktriangle , \triangle), [¹²⁷I]PTA-OH (\blacksquare , \square), and PTA-OH (\blacklozenge , \lozenge). The data are presented as the means of duplicate determinations for four paired experiments per compound. SE bars were omitted for clarity.

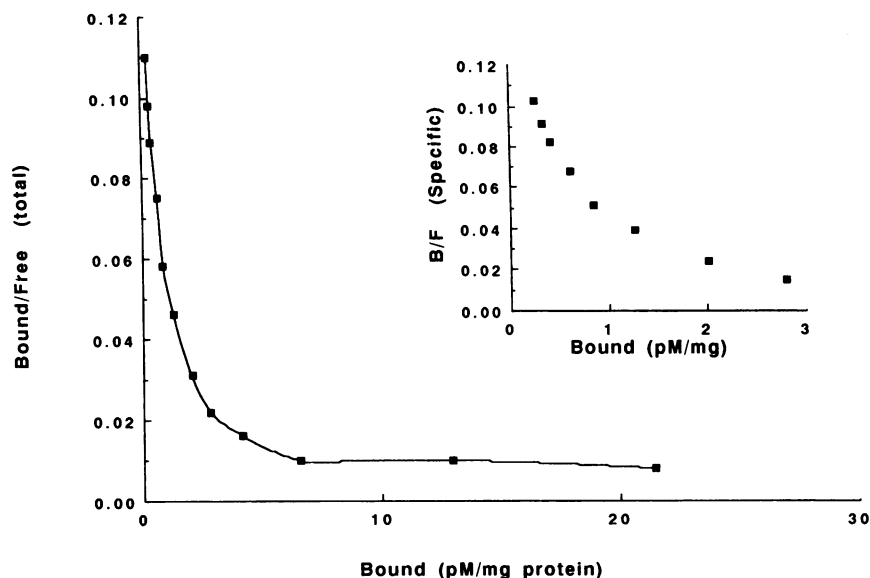


Figure 5. Scatchard plot of the equilibrium binding data of [¹²⁵I]BOP to washed platelets. The data are presented as the means of duplicate determinations for six experiments. The main figure shows the Scatchard plot for total binding. (Inset) Plot when corrected for non-displaceable binding.

cooperativity between sites. Nonlinear curve fitting of the combined data was most consistent with a two-site model ($P < 0.025$ versus a single-site model) that identified a high-affinity binding site with a K_d of 0.234 ± 0.103 nM and a binding capacity of 0.7 ± 0.3 pmol/mg protein (180 ± 87 binding sites/platelet) and a lower affinity site with a K_d of 2.31 ± 0.86 nM and a binding capacity of 2.2 ± 0.3 pmol/mg protein (666 ± 65 binding sites/platelet). When the competition curves were analyzed individually, a two-site model was significantly better than a single-site model ($P < 0.05$) in four of the six experiments. Addition of the nonhydrolyzable guanine nucleotide analogue Gpp(NH)p (1 mM) to the assay did not appear to have any effect ($n = 2$, data not shown).

The K_d of [¹²⁵I]BOP was also determined kinetically. The association rate constant (k_1) for the ligand-binding site complex was determined from the time course of binding of [¹²⁵I]-BOP. At a concentration of 0.1 nM binding appeared to approach equilibrium within 30 min (Fig. 6). The observed rate constant (k_{obs}) derived from the slope of the pseudo first-order rate plot was 0.076 min^{-1} . The dissociation rate constant (k_{-1}) was determined from the time course of displacement of [¹²⁵I]BOP from its binding site induced by an excess of [¹²⁷I]-

BOP. An exponential decrease in binding was observed (Fig. 7). Linear transformation of the data (Fig. 5, inset) yielded a k_1 of 0.032 min^{-1} . The association rate constant determined from the equation $k_1 = (k_{obs} - k_{-1})/[L]$ was found to be $4.4 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$. The kinetically determined dissociation constant (K_d) is given by $K_d = k_{-1}/k_1$ and was found to be 0.073 nM. To confirm these results the association and dissociation rate constants were independently determined from the plot of the observed association rate constant for three different concentrations of [¹²⁵I]BOP as a function of [¹²⁵I]BOP concentration. The slope of this plot (Fig. 6, inset) is k_1 ($3.32 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$) and the Y intercept is k_{-1} (0.052 min^{-1}). The dissociation constant (K_d) is again given by $K_d = k_{-1}/k_1$ and was 0.157 nM.

To differentiate between the possibility of either two unrelated [¹²⁵I]BOP binding sites or negative cooperativity in a single receptor population the time course of displacement was also studied using a different technique. [¹²⁵I]BOP was associated to washed platelets in a manner identical to the above studies but dissociation was initiated by diluting aliquots of the platelet preparation 100-fold without addition of excess unlabeled ligand. At these dilute concentrations of platelets and

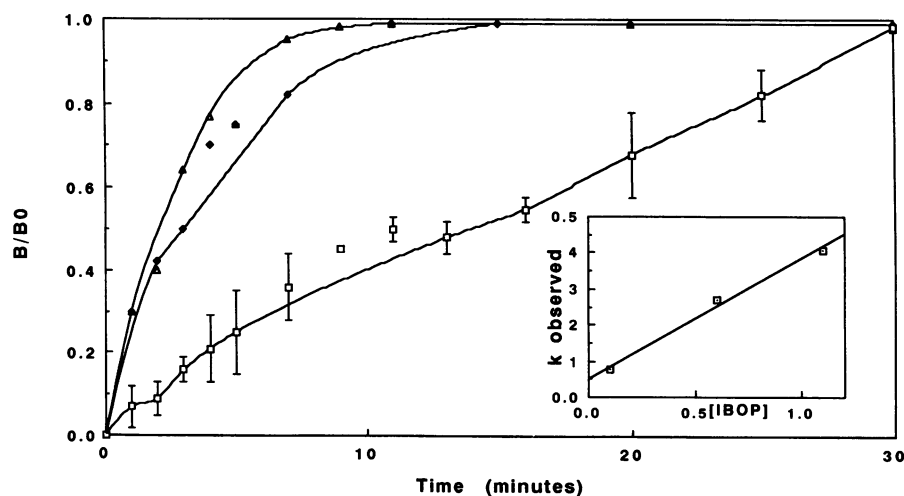


Figure 6. Time courses of association of [¹²⁵I]BOP to washed platelets at 20°C. Each point is the mean \pm SEM of three (0.1 nM, \square) or mean of a single (0.6 nM, \blacklozenge ; 1.1 nM, \triangle) experiment with duplicate determinations. The data were analyzed assuming a pseudo-first order process and their slopes ($k_{observed}$) were plotted as a function of I-BOP concentration (inset). The slope of the plot is the association rate constant (k_1) and the y-intercept is the dissociation rate constant (k_{-1}).

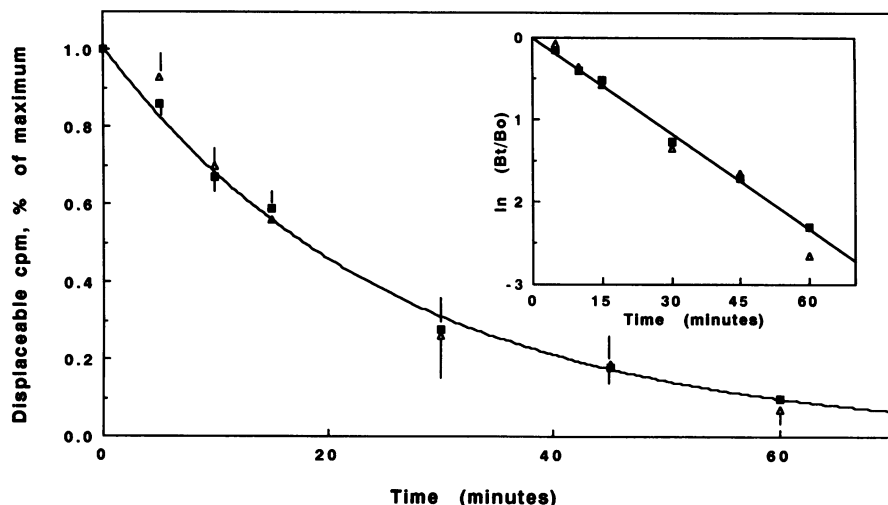


Figure 7. Time courses of dissociation of [125 I]BOP from washed platelets at 20°C. The squares represent dissociation of the ligand-binding complex induced by the addition of 1 μ M [127 I]BOP and the triangles represent dissociation induced by 100-fold dilution, both plotted as a function of time. The data are presented as the means \pm SEM of two experiments performed with duplicate determinations. (Inset) Logarithmic replot, the negative slope of which is the dissociation rate constant (k_{-1}).

radioligand no association of the ligand occurred. Again, an exponential binding decay curve occurred very similar to the previous displacement curve (Fig. 6) and linear transformation of this curve (Fig. 7, inset) resulted in a k_{-1} of 0.044 min^{-1} , similar to the value obtained by displacing with unlabeled ligand.

Binding of [125 I]PTA-OH to washed platelets. $\text{TXA}_2/\text{PGH}_2$ antagonist competition curves for [125 I]BOP and [125 I]PTA-OH are shown in Fig. 4. In contrast to the equilibrium binding studies of [125 I]BOP, the Hill plot for [127 I]PTA-OH to inhibit [125 I]PTA-OH binding was of normal steepness with a Hill coefficient in six experiments of -1.08 ± 0.04 (not significantly different from unity) and an IC_{50} of 16 ± 2 nM. The Scatchard plot was linear (Fig. 8, inset) and nonlinear curve fitting of the combined data from six experiments indicated the best fit was to a single-site model ($P < 0.001$ vs. a two-site model) with a K_d of 10 ± 1.3 nM and a binding capacity of 6.5 ± 0.7 pmol/mg protein ($2,009 \pm 216$ sites/platelet).

Displacement of [125 I]BOP and [125 I]PTA-OH binding by $\text{TXA}_2/\text{PGH}_2$ agonists and antagonists. Competition for [125 I]BOP and [125 I]PTA-OH binding to washed platelets was compared using a number of analogues with $\text{TXA}_2/\text{PGH}_2$ receptor

agonist or antagonist activities in platelets (Figs. 3 and 4, respectively). The agonists were significantly more potent in displacing the agonist ligand [125 I]BOP than the antagonist ligand [125 I]PTA-OH (Table I). As noted above, the Hill coefficient for [127 I]BOP to displace [125 I]BOP was low but the Hill coefficient for [127 I]BOP to displace [125 I]PTA-OH was not significantly different from unity (Table I).

Three antagonists were characterized in the same manner. Hill coefficients were not significantly different from unity for antagonist competition of either the agonist or antagonist ligand (Table I). The two most potent of these compounds, [127 I]PTA-OH and SQ29,548, were significantly less potent competitors for the $\text{TXA}_2/\text{PGH}_2$ receptor agonist binding site compared with the antagonist binding site (Table I).

Discussion

This study provides evidence for two distinct $\text{TXA}_2/\text{PGH}_2$ receptor subtypes in human platelets. [125 I]BOP, the most potent radiolabeled $\text{TXA}_2/\text{PGH}_2$ agonist yet described (14) was used in equilibrium binding studies to washed human platelets. [125 I]BOP appears to have bound to the putative platelet

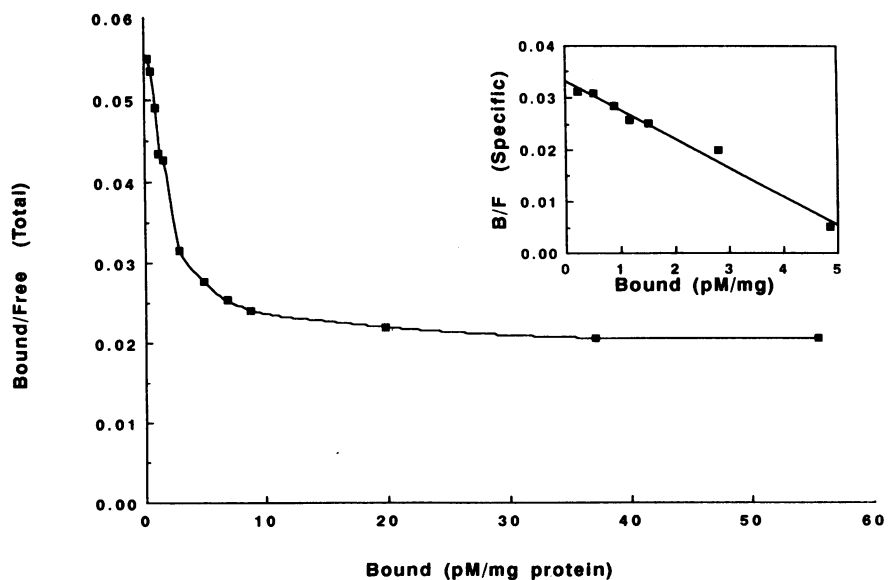


Figure 8. Scatchard plot of the equilibrium binding data of [125 I]PTA-OH to washed platelets. The data are presented as the means of duplicate determinations of six experiments. The main figure shows the Scatchard plot for total binding. (Inset) Plot when corrected for nondisplaceable binding.

Table I. TXA₂PGH₂ Agonist and Antagonist Displacement of [¹²⁵I]BOP and [¹²⁵I]PTA-OH

	[¹²⁵ I]BOP		[¹²⁵ I]PTA-OH	
	IC ₅₀ nM	Hill coefficient (n)	IC ₅₀ nM	Hill coefficient (n)
Agonists				
[¹²⁷ I]BOP	0.94±0.18*	-0.73±0.05**‡ (6)	11±1	-1.01±0.11 (4)
U46619	17±2*	-0.90±0.07 (4)	82±14	-0.94±0.09 (4)
BOP-amine	39±7	-0.74±0.13 (4)	331±63	-1.43±0.24 (4)
Antagonists				
[¹²⁷ I]PTA-OH	36±2*	-0.94±0.03 (4)	16±2	-1.08±0.04 (6)
PTA-OH	240±36	-0.94±0.08 (4)	149±54	-0.95±0.04 (4)
SQ29,548	15±2*	-0.87±0.11 (4)	7±2	-0.97±0.14 (4)

* Different from same value for [¹²⁵I]PTA-OH ($P < 0.05$). ‡ Different from unity ($P < 0.001$).

TXA₂/PGH₂ receptor because it was completely displaced from its binding site by a series of TXA₂/PGH₂ analogues with a rank order identical for these agents to displace the TXA₂/PGH₂ receptor antagonist [¹²⁵I]PTA-OH. Equilibrium binding studies with [¹²⁵I]BOP resulted in competition curves with Hill coefficients which were significantly less than unity and Scatchard plots, which were concave upward. Nonlinear analysis of the binding isotherms fitted the data best to a two-site model: a higher affinity site with a K_d of 234 pM and a lower affinity site with a K_d of 2.3 nanomolar in a molar ratio of ~ 1:4. The existence of a high-affinity site was confirmed by kinetic studies of [¹²⁵I]BOP binding that resulted in K_d estimates of 73 and 157 pM.

The potency of I-BOP to stimulate platelet function closely paralleled its affinity for the two binding sites. The EC₅₀ for I-BOP to promote platelet aggregation was 4.4 nM, similar to the lower affinity K_d of 2.3 nM. However, the EC₅₀ for shape change was 263 pM, similar to the higher affinity K_d of 234 pM. Although both platelet aggregation and shape change are calcium dependent phenomena, shape change is dependent on the early redistribution of intracellular calcium stores, whereas aggregation requires the influx of extracellular calcium. Therefore, the initial increase in intraplatelet calcium ([Ca]_i) was assessed simultaneously with the platelet function studies using the photoprotein aequorin. The EC₅₀ for I-BOP to induce the initial [Ca]_i was 209 pM, which correlates well with the 263-pM EC₅₀ for shape change and also approximated the high-affinity [¹²⁵I]BOP K_d . If one assumes that the I-BOP-receptor interaction follows mass-action law and that the relationship of receptor occupancy to platelet response is linear, then these findings suggest that the two putative receptors may independently mediate platelet aggregation and shape change. This schema is consistent with the notion that there are separate platelet TXA₂ and endoperoxide (PGH₂) receptors (3, 4) with distinct functions. As suggested by Carmo et al., endoperoxides may be responsible for irreversible platelet aggregation and secretion whereas TXA₂ may cause initial platelet shape change and cellular approximation (3).

A previous study of [¹²⁵I]BOP binding to washed human platelets by Morinelli et al. reported a single K_d of 2.2 nM by Scatchard analysis (14). Hill coefficients were not reported. The failure to observe a high-affinity binding site by Scatchard analysis in that study may result from differences in technique

or their use of Tris buffer, which is known to affect platelet function (19, 20).

Studies of human platelet TXA₂/PGH₂ receptor binding with the endoperoxide agonist [³H]U46619 have demonstrated a single binding site with reported K_d s that range from 20 to 131 nM (6–8). Because the lowest reported K_d for U46619 is roughly two orders of magnitude greater than the K_d of the higher affinity I-BOP binding site, [³H]U46619 may not be sufficiently potent to recognize that site. Additionally, if the high-affinity site represents a specific receptor for TXA₂ and the lower affinity site is an endoperoxide receptor as suggested above, then the endoperoxide analogue U46619 might not recognize the high-affinity receptor.

In this study, as in previous studies (9, 10), binding of the radioiodinated TXA₂/PGH₂ receptor antagonist [¹²⁵I]PTA-OH failed to show any evidence of complex binding phenomena or multiple binding sites. One explanation for this seeming inconsistency may be that [¹²⁵I]PTA-OH, with a K_d of 10 nM, lacks sufficient affinity to discriminate between the binding sites. However, a more likely possibility is that its properties as an antagonist differ such from the agonist I-BOP that it does not distinguish between the two receptors. Fundamental differences in binding characteristics between agonists and antagonists have been demonstrated using the β -adrenergic receptor as a model system (13). Antagonists bind with less specificity than do agonists and, in general, with greater capacity. Whereas the antagonist-receptor interaction is thermodynamically favorable, the agonist-receptor interaction is thermodynamically unfavorable but results in a conformational change in the receptor-ligand complex which permits the subsequent cellular response(s) (12). The antagonist lacks the proper "fit" in the receptor to induce the appropriate conformation and thus no response occurs (13). Therefore, it is not surprising that differences exist in the binding characteristics of the two ligands used in this study.

Low Hill coefficients and curvilinear Scatchard plots do not result exclusively from the presence of multiple receptors. Negative cooperativity within a single receptor population, where receptor affinity increases as the proportion of occupied receptors decreases, also results in concave upward Scatchard plots and low Hill coefficients. One way to distinguish between these two phenomena is to displace the ligand from its binding site using dilution instead of adding unlabeled competitor (21).

If negative cooperativity were present the slope of the dissociation curve would be more shallow in the dilution experiment than the competition experiment because the ligand affinity of the receptor increases as a greater proportion of receptor is left unoccupied. This would not be the case for multiple binding sites where the affinity of each independent site does not change with receptor occupancy. In this study dissociation by both competition and serial dilution demonstrates nearly identical dissociation curves. The data strongly suggest that two different receptor populations rather than negative cooperativity is the explanation for the observed complex binding phenomena.

An additional finding of the present study is the differences in affinity of the agonist and antagonist compounds at either the agonist or antagonist binding site (represented by [¹²⁵I]-BOP and [¹²⁵I]PTA-OH, respectively). The TXA₂/PGH₂ receptor agonists were more potent at the agonist binding site compared to the antagonist binding site. This is consistent with a previous study in washed human platelets where the K_d of U46619 to displace tritiated U46619 (agonist) was 20 nM but to displace the antagonist [¹²⁵I]PTA-OH was 145 nM (6). Two other studies have noted that the IC₅₀ for U46619 to displace [¹²⁵I]PTA-OH in washed human platelets was 84 (9) and 148 nM (22). These published IC₅₀ values for U46619 to displace a TXA₂/PGH₂ receptor agonist and antagonist approximate the IC₅₀ for U46619 to displace I-BOP (17 nM) and I-PTA-OH (82 nM) reported herein. These data and this study suggest that TXA₂/PGH₂ mimetics bind to sites on human platelets only partially recognized by the antagonist I-PTA-OH.

In addition to demonstrating differences in TXA₂/PGH₂ receptor agonist binding, the present study also demonstrated differences in the affinities of TXA₂/PGH₂ receptor antagonists for the agonist or antagonist binding site. SQ29,548 and I-PTA-OH were significantly more potent displacing the radioiodinated antagonist compared with the agonist. This suggests that the antagonists are binding in part to a site that is unrecognized by agonists. The heterogeneity of binding of these compounds suggests that the development of TXA₂/PGH₂ analogues specific for the high- and low-affinity platelet binding site may be possible.

The potential significance of these results may relate to the development of receptor antagonists that are specific for the putative endoperoxide receptor. In clinical conditions of intravascular thrombus formation, selective blockade of platelet endoperoxide receptors would prevent platelet aggregation and thrombus formation but would still allow for platelet-endothelium interactions to occur through platelet adhesion resulting from stimulation of the thromboxane receptor. Maintenance of platelet adhesion to vascular endothelium could allow for continued synthesis, by endothelial cells, of prostacycline generated from endoperoxides released by contiguous platelets (23, 24). Thus, the clinical use of specific platelet endoperoxide receptor antagonists has theoretical advantages over the use of agents such as cyclooxygenase inhibitors, which in addition to inhibiting endoperoxide and thromboxane synthesis also suppress formation of prostacycline, thromboxane synthesis inhibitors that block TXA₂ but not endoperoxide formation, and nonspecific TXA₂/PGH₂ receptor antagonists that would inhibit platelet-vessel wall interactions as well as platelet aggregation.

In summary, a new radioiodinated TXA₂/PGH₂ receptor

agonist, I-BOP, was used in radioligand binding studies to washed human platelets. Evidence is presented that this agent bound to two independent platelet TXA₂/PGH₂ receptors. Parallel functional studies of platelet aggregation and shape change/intracellular calcium flux suggest that the two putative receptors may independently mediate these platelet responses. Therefore, these two receptors may represent the separate TXA₂ and endoperoxide receptors predicted by Carmo. Further studies of human TXA₂/PGH₂ receptor subtypes and the possibility of distinct platelet TXA₂ and endoperoxide receptors are underway but definitive proof for the existence of receptor subtypes may require the development of selective ligands and, eventually, purification of the receptor(s).

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