Biochemical Pharmacology, Vol. 44, No. 3, pp. 495–503, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

BINDING CHARACTERISTICS OF THE NEW THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR ANTAGONIST [^{3}H]BAY U 3405 TO WASHED HUMAN PLATELETS AND PLATELET MEMBRANES

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(*Received* 25 *July* 1991; *accepted* 23 *April* 1992)

Abstract—The new thromboxane A_2 antagonist [^{3}H]BAY U 3405 was characterized for its binding to washed human platelets and platelet membranes. In washed platelets the specific binding was reversible, selective and stereospecific, but not saturable. The dissociation constant (K_d) was 6 ± 2.5 nM, the number of specific binding sites 1177 ± 306 per platelet. Three structurally different thromboxane A_2 (TXA_2)/prostaglandin H_2 (prostaglandin endoperoxide) (PGH_2) receptor ligands completely inhibited the specific binding of [^{3}H]BAY U 3405 in a concentration-dependent manner, indicating that the observed high affinity binding site is the TXA_2/PGH_2 receptor. In platelet membranes, however, specific [^{3}H]BAY U 3405 binding showed saturability in addition to reversibility, selectivity, and stereospecifity. The K_d of the binding was 9.6 ± 2.3 nM in kinetic studies and 8.7 ± 3.7 nM in saturation studies, the inhibition constant (K_i) was 10 ± 1.1 nM in displacement studies. The TXA_2/PGH_2 receptor agonists U 46619 and CTA_2, and the antagonists Daltroban (BM 13505), I-PTA-OH and SQ 29548 all completely inhibited the specific binding of [^{3}H]BAY U 3405 thus defining the observed binding site as the TXA_2/PGH_2 receptor. In conclusion, the data suggest that the previously reported TXA_2 antagonism of BAY U 3405 is mediated by binding to a specific high affinity binding site of human platelets and platelet membranes that represents the TXA_2/PGH_2 receptor.

Thromboxane A_2 (TXA_2†) is a potent naturally occurring stimulator of platelet aggregation and constrictor of vascular and bronchial smooth muscles [1, 2]. Similar effects are seen with the precursor of TXA_2, prostaglandin endoperoxide (PGH_2), and are thought to be mediated by a common TXA_2/PGH_2 receptor [3]. The presence of this receptor in platelets, vascular smooth muscle, endothelial cells and lung has been confirmed by a variety of radioligand binding studies [4–15].

TXA_2 is thought to play a pathophysiological role in many cardiovascular, cerebrovascular and pulmonary diseases such as angina, stroke and bronchial asthma [16–18].

BAY U 3405, a new thromboxane antagonist (Fig. 1), prevented platelet aggregation induced by U 46619, arachidonic acid or collagen in human platelet-rich plasma. In contrast, aggregation evoked by quantities of ADP which induce monophasic aggregation was not affected [19]. Moreover, BAY U 3405 inhibited the contraction of different vascular and bronchial smooth muscle preparations induced by TXA_2/PGH_2, CTA_2 and U 46619. The constrictive action of non-prostanoid agonists was not affected [20, 21]. Therefore, the TXA_2/PGH_2 antagonism of BAY U 3405 was thought to be mediated by a blockage of the TXA_2/PGH_2 receptor.

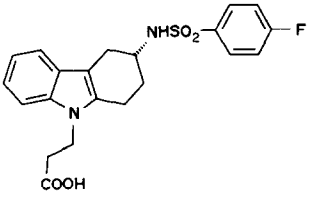

Fig. 1. Chemical structure of BAY U 3405.

This study demonstrates the specific binding of BAY U 3405 to the TXA_2/PGH_2 receptor and compares its binding to washed human platelets and platelet membranes.

MATERIALS AND METHODS

Materials

The synthesis of the (+)-enantiomer [^{3}H]BAY U 3405, (3*R*)-3-(4-fluorophenyl-sulfonamido)-1,2,3,4-tetrahydro-9-carbazolepropanoic acid, having a specific activity of 7.9 Ci/mmol, BAY U 3405, and the (-)-enantiomer BAY U 3406 has been described before [22, 23]. The TXA_2/PGH_2 receptor agonists CTA_2 and U 46619 were obtained from Prof. Nicolaou (University of Pennsylvania, PA,

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† Abbreviations: TXA_2 (B_2), thromboxane A_2 (B_2); CTA_2, carboxylic thromboxane A_2; PGH_2, prostaglandin H_2, prostaglandin endoperoxide; PGD_2 (E_1, E_2, $F_{2\alpha}$), prostaglandin D_2 (E_1, E_2, $F_{2\alpha}$); ACD, acid citrate dextrose.

U.S.A.) and Cayman (Ann Arbor, MI, U.S.A.), respectively. The TXA_2/PGH_2 receptor antagonists Daltroban (BM 13505) and SQ 29548 were generous gifts of Boehringer (Mannheim, Germany) and Squibb (Princeton, NJ, U.S.A.). The TXA_2/PGH_2 receptor antagonist I-PTA-OH was purchased from NEN (Bad Homburg, Germany). The prostaglandins D_2, E_1, E_2 and $F_{2\alpha}$ and Indomethacin were purchased from Sigma (München, Germany). All other reagents were of analytical grade. Human buffy-coat was obtained from a local blood bank.

Preparation of washed human platelets

Blood (100 mL) was collected by venipuncture into 8.3 mL acid citrate dextrose (ACD) (38 mM citric acid, 75 mM trisodium citrate, 122 mM glucose) containing 10 μM Indomethacin. It was assumed that the donors had not taken any form of medication during the last 10 days prior to the study. The blood was immediately centrifuged at 130 *g* for 20 min at room temperature. The resulting platelet-rich plasma was adjusted to pH 6.5 by adding ACD and was recentrifuged at 900 *g* for 20 min. The supernatant was discarded and the platelets were suspended in washing buffer (12 mM Tris–HCl, 139 mM NaCl, 1.5 mM EDTA, pH 7.4). The platelets were washed twice by centrifugation at 900 *g* for 20 min. The washed platelets were finally resuspended in assay buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM glucose, pH 7.4) containing 10 μM Indomethacin. Contaminating red blood cells were removed by centrifugation at 100 *g* for 5 min. The platelet concentration wad adjusted to 5×10^8 platelets/mL.

Preparation of platelet fractions and membranes

Buffy-coat was prepared by centrifugation of human blood, not older than 20 hr, at 2500 *g* for 10 min at 10°. Indomethancin (10 μM) was added to the buffy-coat and the erythrocytes and white blood cells were removed by centrifugation at 175 *g* for 30 min at room temperature. The supernatant—concentrated platelet-rich plasma—was adjusted to pH 6.5 by adding ACD. The platelets were centrifuged off at 1570 *g* for 30 min and resuspended in washing buffer. Contaminating red blood cells were removed by centrifugation at 175 *g* for 5 min and the platelets were washed at 1570 *g* for 20 min. The decontamination and washing procedure was repeated once. The resulting washed platelets were suspended in a small quantity of washing buffer.

The platelet membranes were isolated by a modified method according to Barber and Jamieson [24]: the intracellular glycerol concentration of the platelets was raised to 4.3 M by a very slow centrifugration through glycerol gradients (38 mL, 0–40% glycerol in washing buffer) at 1090 *g* for 30 min at 20° followed by 5010 *g* for 10 min. The following steps were performed at 4°. The supernatant was removed and the platelets were suspended in five times their volume of Tris–sucrose buffer (25 mM Tris–HCl, 0.25 M sucrose, pH 7.4) and simultaneously lysed by rapid mixing of each tube on a vibro mixer and six strokes with a Dounce homogenizer. The suspension was layered on top of a sucrose solution [27% (w/w) sucrose, 25 mM Tris–HCl, 1 mM EDTA, pH 7.4] and centrifuged in a Beckmann SW 28 swing out rotor at 65,000 *g* for 180 min. The opaque layer between the sucrose layer and the supernatant containing the platelet membranes was collected, diluted with the same amount of Tris–NaCl buffer (50 mM Tris, 100 mM NaCl, 1.5 mM EDTA, pH 7.4) and pelleted in a Beckmann 45 Ti rotor at 106,000 *g* for 60 min. The resulting pellet was suspended in assay buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM glucose, pH 7.4) and washed by centrifugation at 106,000 *g* for 60 min. Finally the membranes were resuspended in assay buffer, quick-frozen in dry ice and stored at −80° until assayed.

Protein determination

Protein content of the membrane suspensions was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.) against bovine serum albumin as a standard.

Binding studies

Binding studies were performed in polystyrene tubes (Sarstedt, Nümbrecht, Germany) at 25° (platelets) or 37° (membranes). Platelets ($0.5–1 \times 10^8$) or 50 μg protein were included in the incubation mixture to make sure that both a sufficient number of counts per minute and a low concentration of receptors in relation to ligand dissociation constants was achieved. At least three samples per determination were used. Non-specific binding was determined in the presence of 1000-fold excess of BAY U 3405. The number of experiments performed with platelet or platelet membranes obtained from different individuals and prepared separately is given with the results or in the legends of the figures. Reactions were terminated by adding 4 mL of ice-cold assay buffer followed immediately by rapid vacuum filtration through glass fiber filters (Whatman, Maidstone, U.K.), GF/C for platelets and GF/F for membranes. The filters were washed three times using 4 mL of the ice-cold assay buffer each time. The whole filtration and washing procedure was completed within 15 sec. The filters were dried under reduced pressure, soaked in 5 mL of Quick Scint 401 (Zinsser, Frankfurt, Germany) and analysed for radioactivity content using a LKB Rackbeta 1219 Liquid Scintillation Counter (Pharmacia, Freiburg, Germany). Background filter binding was usually in the range of 0.2–0.4% of the total radioactivity.

Platelets. For the association experiments 5 nM [^{3}H]BAY U 3405 were incubated with 1×10^8 platelets in a volume of 500 μL for 0.5–90 min.

For dissociation experiments 5 nM [^{3}H]BAY U 3405 were incubated with 1×10^8 platelets in a volume of 400 μL for 90 min. Then, the first set of aliquots was filtered to define the total binding at the zero time point. BAY U 3405 in 100 μL assay buffer was added to the other vials to achieve a concentration of 5 μM and to start the dissociation reaction. The dissociation reactions were interrupted by filtration at selected time points.

Saturation experiments were performed by incubating various concentrations (1–180 nM) of [^{3}H]BAY U 3405 and 5×10^7 platelets for 40 min in 250 μL assay buffer. This time of incubation was

chosen because equilibrium binding had been achieved after this period in experiments involving platelet membranes resulting in low non-specific binding. Most of the additional binding in intact platelets measured after this period might be non-specific binding.

For displacement experiments, 1×10^8 platelets were incubated with 5 nM [^{3}H]BAY U 3405 and increasing concentrations of different ligands in 500 μL assay buffer for 100 min. In these experiments the time of incubation had to be increased since the time to reach equilibrium is prolonged by the presence of inhibitor [25].

Platelet membranes. For the association experiments 5, 10, or 50 nM [^{3}H]BAY U 3405 were incubated with 50 μg membrane protein for 0.5–90 min in 250 μL assay buffer.

For dissociation experiments membranes were incubated with 5 nM [^{3}H]BAY U 3405 for 60 min to allow equilibrium to be achieved. Then, the reaction mixture was divided into aliquots of 250 μL containing 50 μg of protein. The first aliquots were filtered immediately to define the total binding at the zero time point. In one set of experiments, 10 mL of assay buffer were added to the remaining aliquots to start the dissociation reaction. In another set of experiments, BAY U 3405 in 50 μL assay buffer was added to achieve a final concentration of 50 μM. The dissociation reactions were interrupted by filtration at selected time points.

Saturation experiments were performed by incubating various concentrations (1–100 nM) of [^{3}H]BAY U 3405 and 50 μg protein of membranes for 60 min in 250 μL assay buffer.

For inhibition experiments various concentrations of non-labelled compounds were incubated with 5 nM [^{3}H]BAY U 3405 and 50 μg membrane protein in 1.2 mL for 60 min.

Calculations were based on the results of at least three independent experiments unless otherwise indicated. The kinetic data were analysed as described by Weiland and Molinoff [25]. Equilibrium binding data were analysed according to Scatchard [26], Weiland and Molinoff [25] and Mendel and Mendel [27]. Scatchard analysis and displacement curves were tested for significance of fit using the computer program LIGAND [28]. All values are given as means ± SD.

RESULTS

Platelets

The results of the kinetic experiments are shown in Fig. 2. Equilibrium binding was nearly established after 60 min. Dissociation experiments showed slow dissociation and reversibility of the specific binding of [^{3}H]BAY U 3405. Due to the short time of incubation, especially in the dissociation experiments, a quantitative analysis of the data could not be performed.

Saturation binding data and their analysis according to Scatchard [26] are shown in Fig. 3. Between 5 and 30 nM [^{3}H]BAY U 3405, about 40% of the total binding was not displaced and was thus considered non-specific. The specific binding, the difference between total and non-specific binding, was not saturable. In order to examine whether multiple specific binding sites could account for the observed non-saturability, the data were analysed by non-linear least-squares regression analysis using the program LIGAND [28]. Various binding models were compared statistically.

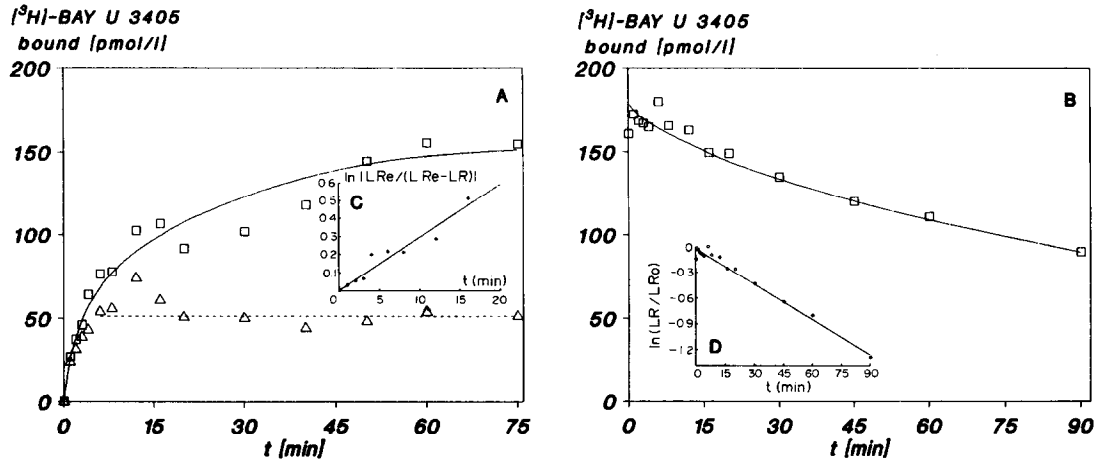

Fig. 2. Time course of association and dissociation of [^{3}H]BAY U 3405 in washed human platelets at 25°. Each point represents the mean of two experiments with triplicate determinations. (A) Association: total binding (□) was obtained by incubation of 5 nM [^{3}H]BAY U 3405 and 1×10^8 platelets, non-specific binding (△) was determined by including 5 μM BAY U 3405 in the incubation mixture. (B) Dissociation: after incubation of 5 nM [^{3}H]BAY U 3405 and 1×10^8 platelets for 60 min, 5 μM BAY U 3405 were added to initate dissociation. (C) Linear transformation of the association data corrected for specific binding according to a pseudo-first order equation. (D) Linear transformation of the dissociation data.

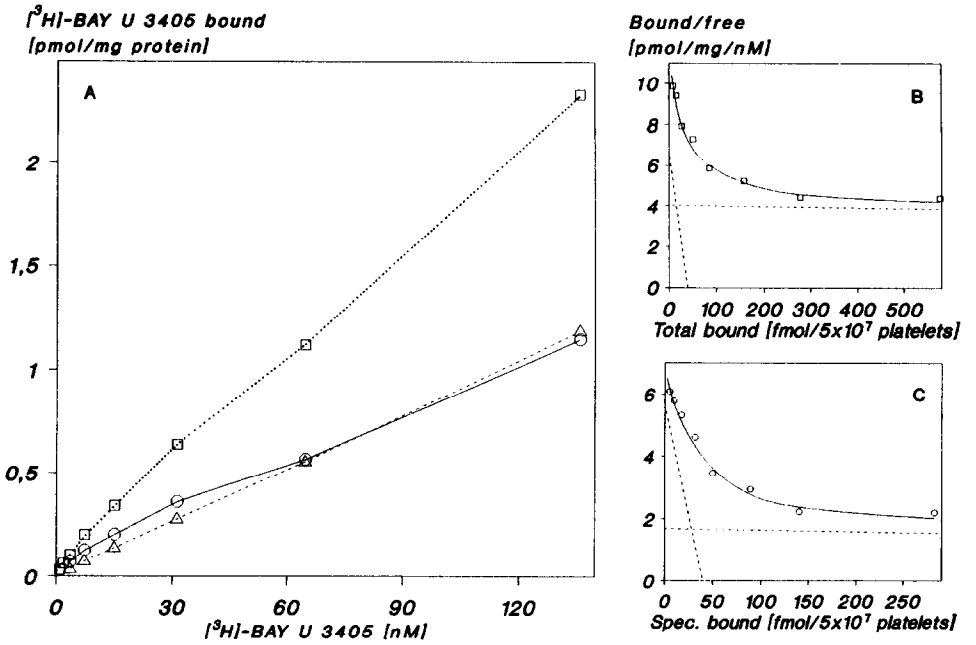

Fig. 3. Saturation binding studies of [^{3}H]BAY U 3405 to washed human platelets. (A) Various concentrations of [^{3}H]BAY U 3405 were incubated with 5×10^7 platelets at 25° for 40 min to obtain total binding (□). Non-specific binding (△) was determined by including a 1000-fold excess of unlabelled BAY U 3405, specific binding (○) is the difference between total and non-specific binding. Each point is the mean of six experiments with quadruple determinations. (B) Scatchard plot of the total binding shown in (A). (C) Scatchard plot of the specific binding in (A). Each dotted line in (B) and (C) represents one population of binding sites as determined by non-linear regression analysis.

Best fit was achieved using only total binding as suggested by Mendel and Mendel [27] (Fig. 3B). This fit resulted in a mean sum of squares of 2.9% compared to at least 5.0% using other models. As this model considers total binding only, the results cannot be influenced by false experimental determination of non-specific binding. A single population of high affinity receptors with a K_d of 6 ± 2.5 nM and a number (B_{max}) of 471 ± 193 binding sites/platelet, and a second population of low affinity binding sites (K_d = 3303 nM, B_{max} = 160,000 binding sites/platelet) were obtained, the latter representing non-specific binding.

Fitting the data, which were corrected for specific binding by substrating the non-displaceable binding from total binding, to a two-site model [28] yielded a high affinity receptor site with almost the same affinity and concentration of binding sites (K_d = 7 ± 3 nM, B_{max} = 475 ± 214 binding sites/platelet) as above and a low affinity site (K_d = 1960 nM, B_{max} = 40,900 binding sites/platelet) (Fig. 3C).

To ensure that the observed high affinity binding site was the TXA_2/PGH_2 receptor, we used three reference compounds which are reported to be selective TXA_2/PGH_2 receptor ligands. The agonist U 46619 [3] and the antagonists BAY U 3405, SQ 29548 [10] and Daltroban (BM 13505) [29] completely inhibited the specific binding of [^{3}H]BAY U 3405 in a concentration-dependent manner (Fig. 4). The inhibition constants (K_i) were: BAY U 3405: 11 ± 4 nM, SQ 29548: 28 ± 8 nM, Daltroban (BM 13505): 140 ± 24 nM and U 46619: 556 ± 89 nM. However, the data for U 46619 were significantly ($P < 0.05$) better fitted to a two-site model: the high affinity binding site calculated to be 13% of all binding sites was described by a K_i of 2 nM, the low affinity binding site, 87% of all binding sites, by a K_i of 732 nM.

BAY U 3406, the (-)-enantiomer of BAY U 3405 also completely inhibited the specific binding of [^{3}H]BAY U 3405. However, its K_i 1006 ± 101 nM) was about 100 times higher than the K_i of BAY U 3405, thus demonstrating stereoselectivity of the receptor.

Non-linear regression analysis of the data derived from inhibition of [^{3}H]BAY U 3405 binding by BAY U 3405 revealed a single class of binding sites with a density of 1177 ± 306 binding sites/platelet.

Platelet membranes

Equilibrium binding with 5 nM [^{3}H]BAY U 3405 was established after 30 min (Fig. 5A). Under these conditions the specific binding was almost 90% of the total binding. The association constant (k_{+1}), determined using the second order equation [25], was independent of the ligand concentration (data not presented) with a mean value of $1.5 \pm 0.2 \times 10^5\ M^{-1}\ sec^{-1}$ (six independent experiments). Dissociation experiments showed complete reversibility of the binding of [^{3}H]BAY U 3405 (Fig. 5B). The decrease in the specific binding was exponential and was not affected by the method of

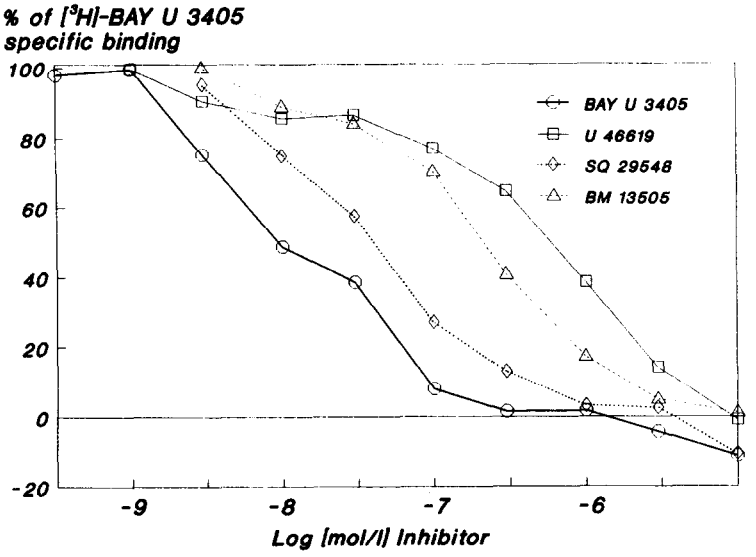

Fig. 4. Inhibiton of the specific binding of [^{3}H]BAY U 3405 to washed human platelets. Various concentrations of the TXA_2/PGH_2 receptor ligands were incubated with 5 nM [^{3}H]BAY U 3405 and 1×10^8 platelets at 25° for 100 min. Specific binding was obtained by substracting non-specific binding obtained with 1 μM BAY U 3405 from total binding. Each point represents the mean of three different experiments with triplicate determinations.

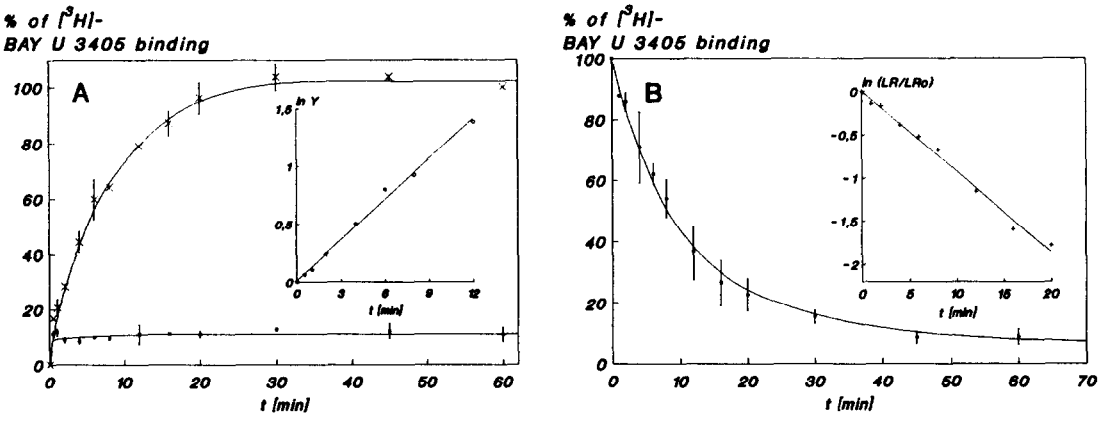

Fig. 5. Time course of association and dissociation of [^{3}H]BAY U 3405 in platelet membranes. Presented is one representative experiment. (A) Association: total binding (×) was obtained by incubation of 5 nM [^{3}H]BAY U 3405 with platelet membranes containing 50 μg protein at 37°, non-specific binding (■) was determined by including 5 μM BAY U 3405 in the incubation mixture. Each point represents the mean of a triplicate determination. Inset: linear transformation of the specific binding data according to the integrated form of the second order rate equation [25]. (B) Dissociation: after incubation of 5 nM [^{3}H]BAY U 3405 and platelet membranes (50 μg protein) for 60 min, 5 μM BAY U 3405 was added to initate dissociation. Each point represents the mean of a triplicate determination. Inset: linear transformation of the specific dissociation data.

starting the dissociation reaction. The dissociation rate constants (k_{-1}), analysed according to a first-order dissociation reaction, were $1.44 \pm 0.15 \times 10^{-3}\,sec^{-1}$ (three independent experiments) after dilution and $1.43 \pm 0.19 \times 10^{-3}$ sec^{-1} (three independent experiments) after displacement by unlabelled BAY U 3405; $T_{1/2}$ was 8.1 min. The kinetically determined K_d was 9.6 ± 2.3 nM.

Specific binding was saturable up to 90 nM (Fig. 6). At ligand concentrations close to the K_d value (5–10 nM) specific binding amounted to 83–90% of total binding. Scatchard analysis of the specific binding resulted in a straight line, indicating a single receptor population with a K_d of 8.7 ± 3.7 nM and a B_{max} of 6.6 ± 0.6 pmol/mg protein. When the specific binding data were tested for two coexisting

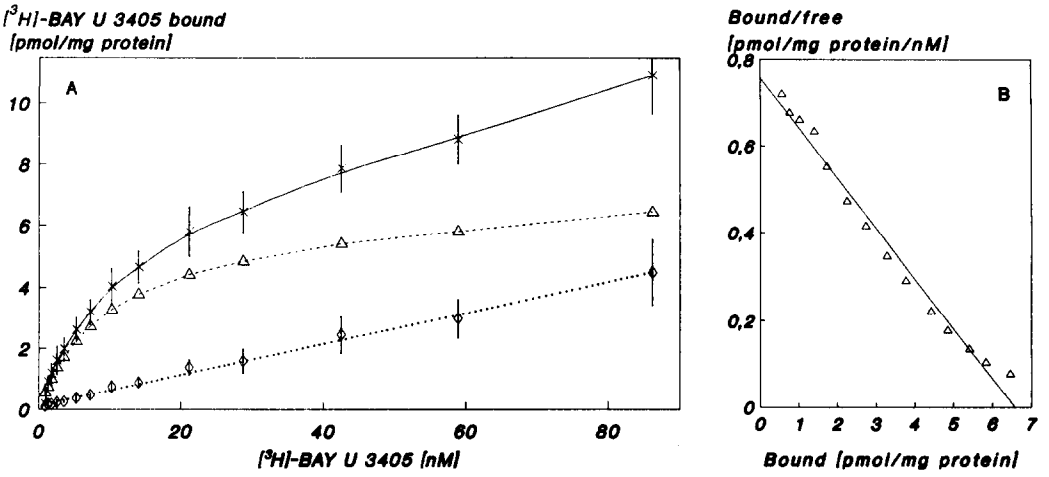

Fig. 6. (a) Saturation of the binding of [^{3}H]BAY U 3405 to platelet membranes. Various concentrations of [^{3}H]BAY U 3405 were incubated with 50 μg membrane protein at 37° for 60 min to obtain total binding (×). Non-specific binding (◇) was determined by including a 1000-fold excess of unlabelled BAY U 3405, specific binding (△) is the difference between total and non-specific binding. The means of seven individual experiments with quadruple determinations ± SD are given. (B) Scatchard plot of the specific binding.

classes of binding sites, a significantly better curve fit was not achieved. Non-linear regression analysis of the total binding according to Mendel and Mendel [27] revealed a population of high affinity binding sites with a K_d of 5.6 ± 3.3 nM and a B_{max} of 5.5 ± 1.7 pmol/mg protein, and low affinity sites which are thought to represent non-displaceable or non-specific binding (K_d = 1181 nM, B_{max} = 1433 pmol/mg protein).

The specific binding of [^{3}H]BAY U 3405 was completely and concentration-dependently inhibited by the TXA_2/PGH_2 receptor antagonists BAY U 3405, SQ 29548 [10], I-PTA-OH [6], BAY U 3406 and Daltroban (BM 13505) [29], and the agonists CTA_2 [30] and U 46619 [3] (Fig. 7). Specific binding was 85–90% of total binding. The K_i values and Hill-coefficients for competitive inhibition of [^{3}H]BAY U 3405 binding are summarized in Table 1. The inhibition by all antagonists resulted in a Hill-coefficient close to unity. In contrast, both agonists showed a Hill-coefficient lower than 0.9.

At a concentration of 100 μM, PGD_2 displaced 66% of specific [^{3}H]BAY U 3405-binding, $PGF_{2\alpha}$ 32%, PGE_2 32%, PGE_1 16% and TXB_2 7%. Up to a concentration of 1 μM the prostanoids and TXB_2 had no effect on the specific binding of [^{3}H]BAY U 3405.

DISCUSSION

Based on the results of pharmacological experiments it was assumed that the TXA_2 antagonism of BAY U 3405 was mediated by specific binding to the TXA_2/PGH_2 receptor [18, 19]. This could be confirmed by demonstrating the binding qualities typical for a ligand–receptor binding such as reversibility, saturability, selectivity and stereospecificity.

Complete reversibility of the specific [^{3}H]BAY U 3405 binding is shown in the kinetic experiments using platelet membranes. Reversibility is also demonstrated in washed platelets. However, it is not fully complete within the observation time due to the slow rate of dissociation.

Saturability of the specific binding of [^{3}H]BAY U 3405 to platelet membranes is observed with ligand concentrations up to 10 times higher than the K_d. Scatchard analysis of the specific binding results in a straight line giving strong evidence for binding to one population of binding sites. In contrast, saturability of the specific binding is not achieved in washed platelets resulting in a curvilinear Scatchard plot. Nevertheless, computerized non-linear regression analysis of the total binding data give a single population of high affinity binding sites.

Selectivity of the binding to the TXA_2/PGH_2 receptor is proven by complete and concentration-dependent inhibition of the specific [^{3}H]BAY U 3405 binding by structurally different TXA_2/PGH_2 receptor ligands in both platelet membranes and intact platelets. Additionally, even high concentrations of PGD_2, $PGF_{2\alpha}$, PGE_1, PGE_2 and TXB_2 have only a little or no effect upon the binding of [^{3}H]BAY U 3405 to platelet membranes.

Stereospecificity of the receptor binding is demonstrated by the large differences in affinity of the two stereoisomers BAY U 3405 and BAY U 3406 to the TXA_2/PGH_2 receptor of both washed platelets and platelet membranes.

In conclusion, all binding qualities typical for a ligand–receptor binding are demonstrated in both washed platelets and platelet membranes with one exception, saturability, which cannot be proven in washed platelets.

Non-saturability of specific binding of TXA_2/PGH_2 receptor ligands to intact platelets has been

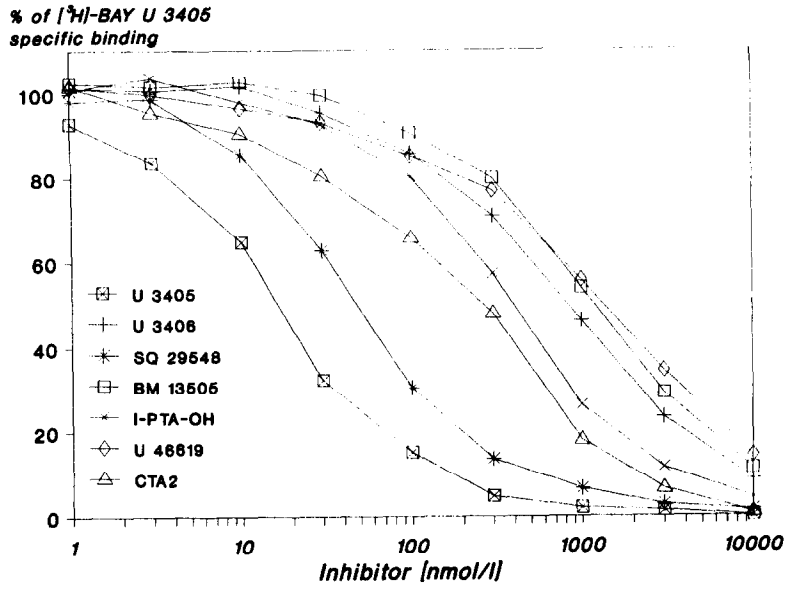

Fig. 7. Inhibition of the specific binding of [^{3}H]BAY U 3405 to platelet membranes. Various concentrations of TXA_2/PGH_2 receptor ligands were incubated with 5 nM [^{3}H]BAY U 3405 and 50 μg membrane protein at 37° for 60 min. The specific binding was obtained by substracting the non-specific binding obtained with 5 μM BAY U 3405 from each total binding. Each point represents the mean of 3–5 different experiments with triplicate determinations.

Table 1. Inhibition of specific [^{3}H]BAY U 3405 binding to platelet membranes by different TXA_2/PGH_2 receptor ligands

Compound	IC_{50} (nM)	K_i (nM)	Hill-coefficient	N
Agonists				
CTA_2	214 ± 75	132 ± 36	0.87 ± 0.10	3
U 46619	850 ± 264	560 ± 243	0.78 ± 0.08	4
Antagonists				
BAY U 3405	16 ± 2	10 ± 1	0.98 ± 0.11	5
SQ 29548	48 ± 6	31 ± 6	1.02 ± 0.17	4
I-PTA-OH	395 ± 102	255 ± 72	0.99 ± 0.02	3
BAY U 3406	794 ± 217	495 ± 96	0.94 ± 0.05	3
Daltroban (BM 13505)	1143 ± 464	768 ± 396	0.95 ± 0.08	5

Various concentrations of the non-labelled ligands were incubated with 5 nM [^{3}H]BAY U 3405 and 50 μg membrane protein as described in Materials and Methods.

The IC_{50} values were determined as the intercept of a Hill plot with the X-axis [31]. The K_i was calculated using the Cheng and Prusoff [32] equation. The Hill coefficient is the slope of the Hill plot for each compound.

The mean ± SD of N experiments is given.

described before using [^{3}H]U 44069 [4] and [^{3}H]U 46619 [9]. The high degree of lipophilicity of these compounds was assumed to contribute to this phenomenon [9].

Both ligands show a high degree of non-specific binding as [^{3}H]BAY U 3405 does in washed platelets. Interestingly, the non-specific binding of [^{3}H]BAY U 3405 is much lower in platelet membranes where saturability can be demonstrated. Saturation studies were also performed with the other subcellular platelet fractions resulting during the preparation of membranes according to Barber and Jamieson [24]. Both saturability and low non-specific binding are seen in all the fractions containing non-soluble proteins (data not presented). This observation suggests that the non-specific binding measured in intact platelets must be [^{3}H]BAY U 3405 trapped inside the platelet or bound to cytosolic proteins. [^{3}H]BAY U 3405 exerts a high non-specific plasma protein binding of 97% (unpublished results).

Non-specific binding is usually determined as the radioligand binding remaining in the presence of an excess of unlabelled ligand. This method of determination is based on the assumption that non-specific binding is non-saturable. However, even ligand uptake or non-specific binding to a limited amount of cytosolic proteins tends to be saturable [27]. As a consequence, the binding must be at least partially inhibited by high concentrations of unlabelled BAY U 3405. Thus part of it is falsely considered specific and specific binding appears non-saturable. This assumption is confirmed by the fact that calculation of the data by a method which does not involve the measurement of non-specific binding [27] results in one population of high affinity binding sites with a K_d close to that determined by other experimental methods. Moreover, in inhibition studies with washed platelets all reference ligands at concentrations only 100-fold higher than their IC_{50} values start inhibiting further (non-specific) binding after having reached a plateau (Fig. 4). Although this may be caused by the existence of an inhibitable uptake system of platelets for TXA_2/PGH_2 receptor ligands as described recently [33], it may also simply represent displaceable non-specific binding.

In conclusion, saturability of the true specific binding site in intact platelets is not contradicted by the experimental data: the apparent non-saturability seems to be caused by radioligand uptake or displaceable non-specific binding with low affinity.

As mentioned above, all data indicate that the TXA_2 antagonist [^{3}H]BAY U 3405 binds to a single population of specific high affinity binding sites. In displacement studies all TXA_2/PGH_2 receptor antagonists inhibit specific [^{3}H]BAY U 3405 binding in a monophasic manner, thus also giving evidence for binding to a single type of receptor. In contrast, the inhibition curves derived from experiments with the agonists U 46619 and CTA_2 results in a more shallow slope for both intact platelets and platelet membranes. Agonists seem to recognize two different receptor populations or rather two affinity states of the same receptor population. This observation may also be explained by down regulation of the affinity or number of TXA_2/PGH_2 receptors by agonists which has been described before [34, 35].

Although the specific binding of [^{3}H]BAY U 3405 is completely inhibited by all of the unlabelled TXA_2/PGH_2 receptor ligands, our observed inhibition constants (K_i) are higher than those determined by inhibition of the radioligands [^{125}I]PTA-OH, [^{3}H]U 46619 or [^{3}H]SQ 29548 [6, 7, 9, 10]. However, our data agree well with those reported by investigators who used [^{3}H]S-145 or [^{3}H]GR 32191 as radioligands [12, 36]. S-1245, GR 32191 and BAY U 3405 all have a slow rate of dissociation compared with the other radioligands.

The number of specific binding sites per platelet (1177) determined by inhibition experiments at equilibrium is well in the range of the number reported by other investigators (550–2530) [4, 6, 7, 9–12]. The number of binding sites determined by saturation studies (471) is lower. This might be due to the difficulties of determining specific binding in saturation studies as described above.

The concentration of TXA_2/PGH_2 receptors in our membrane fraction is higher than that reported by investigators who used different methods to prepare their platelet membranes [5, 8, 10, 12]. Only Saussy *et al.* [37], who used the same method, reported a similar receptor concentration using [^{125}I]PTA-OH as a radioligand, but also reported a low yield of protein in the membrane fraction. By isolating the platelets from large amounts of buffy-coat, which is regarded as waste during the preparation of blood for transfusions, this problem could be solved. These platelets in the buffy-coat and their TXA_2/PGH_2 receptors remained intact and active, as proven by aggregation studies using U 46619, collagen and thrombin (data not presented).

In this study we have demonstrated the binding of [^{3}H]BAY U 3405 to a specific binding site of human platelets, which is shared by other known TXA_2/PGH_2 receptor ligands. The pharmacological equivalent of the binding of BAY U 3405 to the TXA_2/PGH_2 receptor of human platelets is its potency to inhibit aggregation mediated by this receptor. The IC_{50} values of BAY U 3405 for the inhibition of platelet aggregation induced by collagen, arachidonic acid and U 46619 in human plasma are 65, 160 and 700 nM, respectively [19]. These values are at least 10-fold higher than the inhibition constant (K_i) we determined in binding experiments. However, this difference can be explained by the high degree of binding of BAY U 3405 to plasma proteins. In washed human platelets U 46619-induced platelet aggregation is inhibited by lower concentration of BAY U 3405 in the range of 2–24 nM (unpublished data). Thus pharmacological data support that the observed high affinity binding site is coincident with the TXA_2/PGH_2 receptor.

In conclusion, we have demonstrated that the TXA_2 antagonistic effects of BAY U 3405 in platelets are mediated by specific binding to the TXA_2/PGH_2 receptor of platelet membranes and washed human platelets.

Acknowledgements—The authors wish to thank Dr Greiff, Blutspendedienst Breitscheid, for providing the buffy-coat and Dr P. J. Munson and Dr D. Rodbard for providing the computer program LIGAND.

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