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CHARACTERIZATION OF THROMBOXANE A_2/ PROSTAGLANDIN H_2 (TXA_2/PGH_2) RECEPTORS OF RAT PLATELETS AND THEIR INTERACTION WITH TXA_2/PGH_2 RECEPTOR ANTAGONISTS

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Abstract—Characterization of thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors of rat platelets was performed on both intact platelets and crude membrane fractions. The binding of [^{3}H]U46619, a stable TXA_2 mimetic, to intact platelets was found to be saturable and displaceable. Scatchard analysis of equilibrium binding at 24° revealed a single class of binding sites with a K_d of 37 nM and a B_{max} of 160 fmol/10^8 platelets. The binding affinity of [^{3}H]U46619 to the platelet membrane fractions was remarkably and specifically enhanced by addition of Mg^{2+} without alteration of the maximum density level. Kinetic analysis for [^{3}H]U46619 binding to the membrane fractions in the presence of 20 mM $MgCl_2$ gave a K_1 of 6.9×10^6 M^{-1} min^{-1} and a K_{-1} of 0.25 min^{-1}, yielding a K_d (K_{-1}/K_1) of 36 nM; the value corresponded well to K_d values from Scatchard analysis in both intact (37 nM) and crude membrane fractions (39 nM).

A series of TXA_2/PGH_2 receptor antagonists completely suppressed U46619 binding to rat platelets as well as collagen-induced platelet aggregation. The rank order of binding affinities to rat platelets (intact platelets or crude membranes) among the respective antagonists correlated well with (a) that of human platelet membrane fraction and (b) the potencies for suppression of collagen-induced platelet aggregation in rat. These results may support our proposed mechanism of TXA_2/PGH_2 action in collagen-stimulated platelets [K. Hanasaki *et al.*, *Thromb. Res.* **46**, 425 (1987)] and also suggest that they may provide a simple technique for evaluating synthetic TXA_2/PGH_2 receptor antagonists.

Thromboxane A_2 (TXA_2)† is an exceptionally potent inducer of platelet aggregation and constrictor of vascular and respiratory smooth muscle [1–3], and its action has been demonstrated to be elicited via putative receptors on the cell membrane [4]. Since TXA_2 has been postulated to be a mediator contributing to the pathophysiology of a variety of disease processes, such as atherosclerosis, thrombosis and angina [5, 6], a number of compounds which antagonize the actions of TXA_2 have been developed recently as agents of potential clinical value and as tools for defining the physiologic and pathologic roles of TXA_2 [7, 8]. Much of the work on the efficacy and potency of TXA_2/PGH_2 receptor antagonists has focused on platelet aggregation of aliquots of platelet-rich plasma in extravascular aggregometers in the presence of a TXA_2 agonist or an agent that directly activates TXA_2 formation [9]. In a series of 13-azapinane TXA_2/PGH_2 receptor antagonists, Mais *et al.* [10, 11] found a good correlation between their inhibitory potencies for [^{125}I]PTA-OH binding and U46619-induced aggregation in canine or human platelets. However, none of the published reports has definitely shown relationships of the potencies between receptor bindings and antiaggregatory activities of TXA_2/PGH_2 receptor antagonists with different structures.

Some reports have stated that the rat is not an appropriate species to use for studying the role of prostaglandins in platelet aggregation and for developing antithrombotic agents for human use [12, 13], because PGH_2 and U46619, a TXA_2 mimetic agonist [14], can induce only shape change and not aggregation in rat platelets [13, 15]. However, our recent studies on collagen-stimulated rat platelets provided the evidence for a substantial role of TXA_2/PGH_2 in platelet aggregation [16, 17]. A small but significant amount of TXA_2/PGH_2 initially produced by collagen may act as a trigger to provoke subsequent platelet responses (i.e. shape change, aggregation and secretion) under synergism with the receptor occupation by collagen [16]. All the responses were suppressed completely by addition of TXA_2/PGH_2 receptor antagonists, including the sulfonamide BM13177 [18], a 7-oxabicyclo[2.2.1]heptane ring system, SQ29548 [19], a bicyclo[2.2.1]heptane ring system, EP-045 [20], and derivatives of pinane TXA_2 such as ONO11120 [11] and ONO3708 [21]. Comparison of the inhibitory potencies of these antagonists in collagen-induced aggregation of rat platelets with those of TXA_2/PGH_2 receptor binding might, therefore, offer not only further elucidation of our proposed mechanism of TXA_2/PGH_2 action [16, 17], but also serve as a useful model that strictly rep-

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† Abbreviations: TXA_2 (B_2), thromboxane A_2 (B_2); PGH_2 (E_1, D_2), prostaglandin H_2 (E_1, D_2); PGI_2, prostacyclin; PRP, platelet-rich plasma; GFP, gel-filtered platelets; PMSF, phenylmethylsulfonyl fluoride; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

resents their potencies for inhibiting TXA_2/PGH_2 binding to their own receptors.

In the present study, we first characterized the TXA_2/PGH_2 receptor of rat platelets by means of binding studies with radiolabeled U46619, a stable TXA_2 mimetic [14]. We then examined the effect of some TXA_2/PGH_2 receptor antagonists on the platelets to define the availability of rat platelets as a simple primary method to evaluate TXA_2/PGH_2 receptor antagonists.

MATERIALS AND METHODS

Materials. [^{3}H]U46619 (22.4 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Collagen (type IV, soluble), U46619, PGE_1, PGD_2 and bovine serum albumin were purchased from Sigma, St. Louis, MO, U.S.A. TXB_2 was purchased from Funakoshi, Osaka, Japan. Phenylmethylsulfonyl fluoride (PMSF) and EDTA were purchased from Nakarai Chemicals, Kyoto, Japan. BM13177 (4-[2-(benzenesulfonamido)-ethyl]-phenoxyacetic acid), SQ29548 (1S-[1α,2β(5Z), 3β,4α]-7-[3-[2-[(phenylamino)carbonyl]hydrazino]-methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid), EP-045 [(±)5-endo-(6'-carboxyhex-2'Z-enyl-6-exo[*N*-(phenylcarbamoyl)hydrazono-methyl]bicyclo[2.2.1]heptane)], ONO11120 (9,11-dimethylmethano-11, 12-methano-16-phenyl-13, 14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2) and ONO3708 [(9,11), (11,12)-dideoxa-9α, 11α-dimethylmethano-11, 12-methano-13, 14-dihydro-12-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pethanol-15-epi-TXA_2] were synthesized in the Shionogi Research Laboratories, Osaka [21–24]. The structures of these TXA_2/PGH_2 receptor antagonists are shown in Fig. 1. Stock solution of each antagonist was prepared in dimethyl sulfoxide (DMSO), which was stable at −20° for over a month, and diluted in the appropriate buffer before each experiment. The vehicle for [^{3}H]U46619 was the appropriate buffer containing 0.1% DMSO at the final concentration. Other materials and chemicals were obtained from commercial sources. Collagen was solubilized in isotonic saline before use.

Preparation of gel-filtered platelets (GFP). Blood was collected from rat or human volunteers into 0.15 vol. of acid citrate dextrose (85 mM trisodium citrate, 70 mM citric acid and 110 mM glucose) containing 12 μg/ml PGE_1. Platelet-rich plasma (PRP), obtained by centrifugation at 160 *g* for 10 min, was layered on 40% bovine serum albumin [25]. Platelets were sedimented at 1200 *g* for 25 min and resuspended in 0.5 ml of resuspension buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 3.8 mM NaH_2PO_4, 3.8 mM HEPES, 5.6 mM glucose and 0.035% bovine serum albumin, pH 7.35). Platelets were separated from plasma proteins by gel filtration through a column of Sepharose 2B and were suspended in the resuspension buffer.

Preparation of platelet membranes. Platelet membranes were prepared according to the method of Saussy *et al.* [26]. Briefly, platelet pellets were prepared from PRP by centrifugation at 1200 *g* for 15 min and resuspended in ice-cold lysing buffer (5 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 10 μM indomethacin and 0.3 mM PMSF). The platelet suspension was homogenized using ten strokes of a tight-fitting glass Dounce homogenizer at 4°. Platelet membranes were prepared by centrifugation of the homogenate at 30,000 *g* for 12 min at 4°. The resulting pellet was washed twice with lysing buffer. The pellets were resuspended in the incubation buffer (50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 10 μM indomethacin and 0.3 mM PMSF) at a protein concentration of about 10 mg/ml. The suspension was used as platelet membranes and stored at −70° until assay. Protein concentrations were determined by the method of Lowry *et al.* [27].

Measurement of the platelet aggregation. Rat GFP (5×10^8 cells/ml) were preincubated with 1 mM $CaCl_2$ for 2 min at 37° in the presence of various concentrations of TXA_2/PGH_2 receptor antagonists, and then 40 μg of collagen/ml was added. The aggregation and shape change were monitored simul-

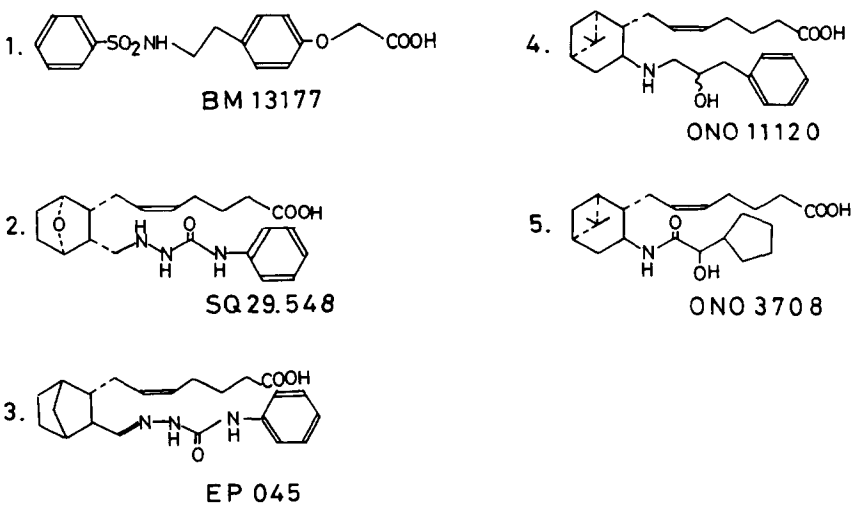

Fig. 1. Structures of TXA_2/PGH_2 receptor antagonists.

taneously with an aggregometer (model PAT-6A, Niko Bioscience Co. Ltd.) in terms of the increase and decrease in light transmission respectively.

Binding studies. Binding studies were performed by incubating 3.4×10^8 cells of GFP or 0.45 mg of platelet membranes with approximately 6 nM [^{3}H]U46619 (0.054 μCi) in a total volume of 0.4 ml in siliconized glass tubes (12 × 75 mm) at 24° for 20 min. Specific binding is defined as the differences between binding in the presence and absence of 10 μM U46619. To block U46619-induced platelet activation, GFP were treated with 1 μM PGI_2 for 2 min prior to mixing them with drugs [28]. After the incubation, ice-cold 0.9% NaCl (3 ml) was added to each tube, and the reaction mixture was immediately filtered by suction through a Whatman GF/C glass filter disc, which was then washed four times with ice-cold 0.9% NaCl. All filtration procedures were completed within 10 sec.

Statistical analyses. Linear regression analysis of Scatchard plots was performed according to the standard methods [29].

RESULTS

[^{3}H]*U46619 binding to gel-filtered rat platelets (GFP).* The binding of 6 nM [^{3}H]U46619 to rat GFP was linear over a platelet concentration range of 1×10^8/ml to 2.5×10^9/ml (data not shown). Total and non-specific binding were not changed significantly by extracellular Ca^{2+} or Mg^{2+} in concentrations from 0 to 10 mM (data not shown). All subsequent binding assays were carried out at pH 7.4 with a platelet concentration of 8.5×10^8/ml and a final concentration of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. This buffer medium exactly duplicated the conditions used in the collagen-induced aggregation studies. Figure 2 displays the time–course of binding and dissociation of [^{3}H]U46619 in rat GFP at 24°. The specific binding, the difference between total and non-specific binding, reached equilibrium within 4 min and remained at the same level for up to 30 min. The addition of a large excess of unlabeled U46619 at the 15-min time point caused rapid and complete displacement of the specifically bound U46619 within 2 min. A saturation curve for [^{3}H]U46619 specific binding is shown in Fig. 3a. The specific binding tended to saturate at a ligand concentration of 200 nM. Scatchard analysis of these data yielded a straight line, indicating a single class of binding sites, with an equilibrium dissociation constant (K_d) of 37 nM and a binding capacity (B_{max})

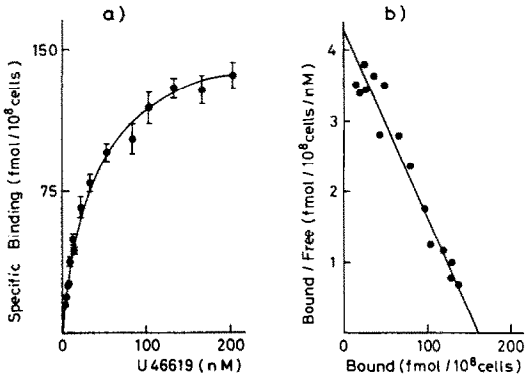

Fig. 3. (a) Saturation of the specific [^{3}H]U46619 binding to rat GFP. GFP were incubated with 6 nM [^{3}H]U46619 in the presence of various concentrations (0–200 nM) of U46619 at 24° for 20 min, and the specific binding was determined by displacing with 10 μM U46619. Mean values are shown; mean ± SE is shown by a vertical line when it exceeds the size of a symbol; (N = 4). (b) Scatchard plot of the specific [^{3}H]U46619 binding to rat GFP. Each point represents the mean value of four experiments shown in (a).

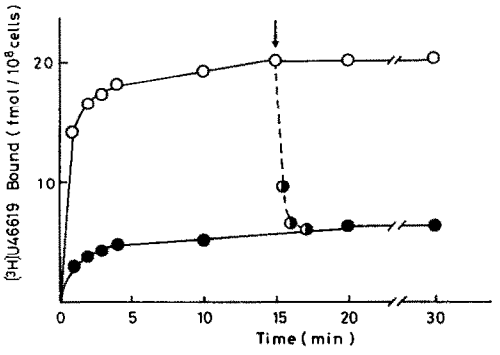

Fig. 2. Time–course of binding and dissociation of [^{3}H]U46619 in intact GFP at 24°. [^{3}H]U46619 (6 nM) was incubated with 3.4×10^8 rat GFP in 400 μl of the resuspension buffer at the indicated times. Total binding (○) was obtained as described in Materials and Methods. Non-specific binding (●) was determined by including 10 μM U46619 in the incubation mixture. After a 15-min incubation, 10 μM unlabeled U46619 was added (arrow) to initiate dissociation of [^{3}H]U46619, and the amount of [^{3}H]U46619 bound was determined after various time intervals (◑, dashed line). This figure is a typical record from one of four similar experiments. Each point represents the mean value of duplicate determinations.

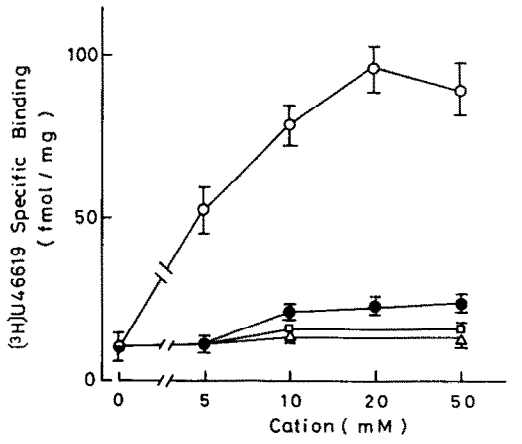

Fig. 4. Effects of cations of specific binding of [^{3}H]U46619 to rat platelet membranes. Rat platelet crude membranes (0.45 mg protein), suspended in the incubation buffer containing 4.5 mM EDTA (final concentration), were incubated with 6 nM [^{3}H]U46619 at 24° for 20 min in the presence of various concentrations of Mg^{2+} (○), Ca^{2+} (●), Na^+ (□) and K^+ (△). The counter ion was chloride for all cations. Mean ± SE calculated from the average of three separate experiments is shown.

of 160 fmol/10^8 platelets, equal to *ca.* 1000 binding sites per platelet (Fig. 3b).

[^{3}H]*U46619 binding to platelet crude membranes.* When 6 nM [^{3}H]U46619 was incubated at 24° with 0.45 mg of crude membranes of rat platelets in the incubation buffer containing a final concentration of 4.5 mM EDTA, specific binding reached a stable, steady-state level after 5 min (data not shown). The effects of various cations on the binding to the crude membranes were determined (Fig. 4). Mg^{2+} enhanced [^{3}H]U46619 specific binding in a concentration-dependent manner, with a maximal stimulatory effect at 20 mM, whereas Ca^{2+} was much less effective than Mg^{2+} and monovalent cations (Na^+ or K^+) did not significantly affect specific binding in concentrations up to 50 mM. To examine the effects of Mg^{2+} on [^{3}H]U46619 binding sites, Scatchard analysis was performed in the presence of 5 or 20 mM Mg^{2+}, because the level of specific binding in the absence of Mg^{2+} was too small to allow an accurate analysis. The Scatchard plot and a computerized analysis indicated the existence of a single class of binding sites with a K_d of 60 or 39 nM and a B_{max} of 750 or 705 fmol/mg in the presence of 5 or 20 mM Mg^{2+} respectively (Fig. 5). These data demonstrated that the density of specific binding sites was not altered significantly, while the affinity of [^{3}H]U46619 binding to the specific sites was enhanced by *ca.* 1.5-fold when Mg^{2+} was increased. In the presence of 20 mM $MgCl_2$, the K_d of [^{3}H]U46619 binding to membrane fractions was almost the same as the value obtained from intact GFP (Fig. 3). Based on these results, all the binding studies using platelet membranes were performed in the presence of 20 mM $MgCl_2$. Under this condition, specific binding of [^{3}H]U46619 to platelet membranes came to equilibrium within 4 min at 24°, which was too fast to perform the kinetical analysis precisely. Thus, the association and dissociation rate constants were determined at 0°. The association rate constant (K_1) for ligand-binding site complex was determined from the time–course of 6.5 nM [^{3}H]U46619 binding. Binding appeared to approach equilibrium within 12 min at 0° (Fig. 6). The observed rate constant (K_{obs}) derived from the slope of the pseudo first-order rate plot (Fig. 6 insert) was found to be 0.295 min^{-1}. To determine the dissociation rate constant (K_{-1}), the membranes were incubated with [^{3}H]U46619 for 20 min at 0° to ensure that equilibrium had been reached. At an arbitrary zero time-

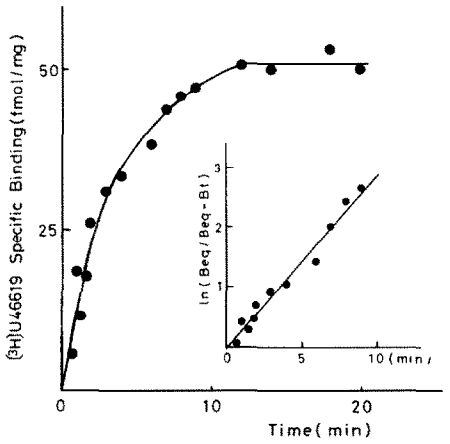

Fig. 6. Time course of association of [^{3}H]U46619 to rat platelet membranes. Rat crude membranes were incubated with 6.5 nM [^{3}H]U46619 in the presence of 20 mM $MgCl_2$ at 0°, and samples were withdrawn at the indicated times. Each point represents the mean value of triplicate determinations. The inset shows the pseudo first-order rate plot of the same data and the slope of the plot is K_{obs}.

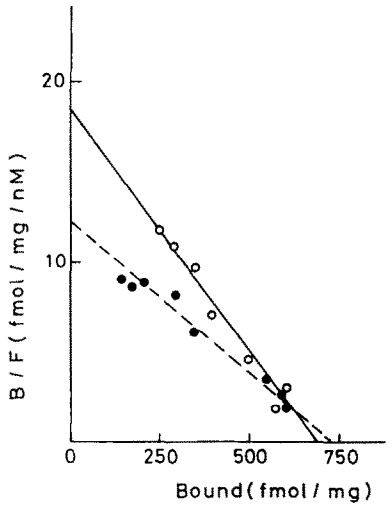

Fig. 5. Scatchard plots of [^{3}H]U46619 binding to rat platelet membranes in the presence of 5 or 20 mM $MgCl_2$. Rat platelet membranes (0.45 mg protein) were incubated at 24° for 20 min with 6 nM [^{3}H]U46619 and various concentrations of unlabeled U46619 (0–200 nM) in the presence of 5 mM $MgCl_2$ (●) or 20 mM $MgCl_2$ (○). Non-specific binding in the presence of 10 μM U46619 was subtracted from each point. Each point represents the mean value of triplicate determinations for three experiments.

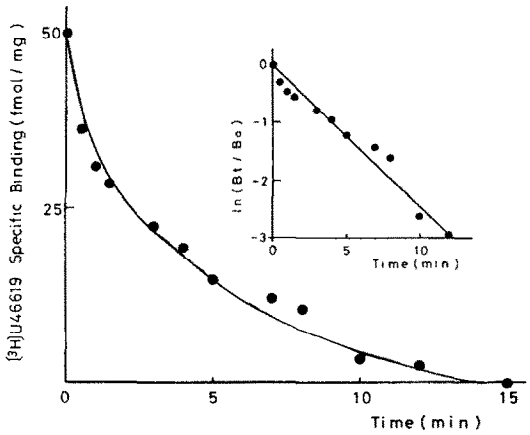

Fig. 7. Time–course of dissociation of [^{3}H]U46619 from rat platelet membranes. Association of rat platelet membranes with 6.5 nM [^{3}H]U46619 in the presence of 20 mM $MgCl_2$ was carried out at 0° for 20 min. Then 10 μM unlabeled U46619 was added to time zero to initiate displacement of [^{3}H]U46619 from its binding site. Each point represents the mean value of triplicate determinations. The inset shows the linear transformation of the same data and the slope of the plot is K_{-1}.

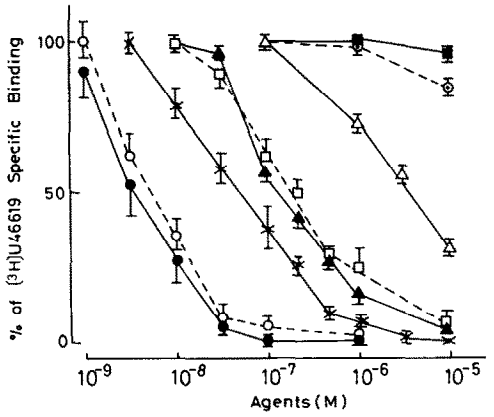

Fig. 8. Displacement of the specific [^{3}H]U46619 binding by various compounds. Rat GFP (3.4×10^8) were incubated with 6 nM [^{3}H]U46619 in the presence of various concentrations of SQ29548 (●), ONO3708 (○), EP-045(×), ONO11120 (▲), BM13177(□), PGD_2 (△), PGE_1 (⊙) and TXB_2 (■). Incubation was carried out at 24° for 20 min. The specific binding was obtained by subtracting non-specific binding obtained with 10 μM U46619 from each total binding. The control value (100%) was defined as the specific binding of [^{3}H]U46619 in the absence of these compounds. Each point represents the mean value ± SE of four experiments.

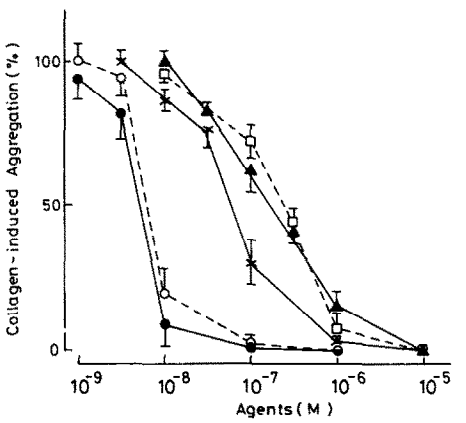

Fig. 9. Concentration–inhibition curves of various TXA_2/PGH_2 receptor antagonists in collagen-induced aggregation of rat GFP. Rat GFP were preincubated with 1 mM $CaCl_2$ for 2 min at 37° in the presence of various concentrations of SQ29548 (●), ONO3708 (○), EP-045 (×), ONO11120 (▲) and BM13177 (□), and then 40 μg of collagen/ml was added. The maximum aggregation reached within 4 min upon addition of collagen was expressed as a percentage of the aggregation induced by collagen in the absence of these compounds. Each point represents the mean value ± SE of four experiments.

point, excess unlabeled U46619 (10 μM) was added, and the time course of displacement of [^{3}H]U46619 from its binding site was determined. As shown in Fig. 7, an exponential decrease in binding was observed and dissociation was complete by 15 min at 0°. Linear transformation of the data revealed a K_{-1} of 0.25 min^{-1} (Fig. 7 insert). The association rate constant was determined to be 6.9×10^6 M^{-1} min^{-1} according to the equation $K_1 = (K_{obs} - K_{-1})/[L]$. The kinetically determined K_d is given by $K_d = K_{-1}/K_1$ and was found to be 36 nM.

Inhibition of [^{3}H]*U46619 binding to rat GFP and crude membranes by* TXA_2/PGH_2 *receptor antagonists.* Several compounds were examined for their abilities to block the [^{3}H]U46619 binding to intact GFP and platelet membranes. Figure 8 shows the displacement curves for these compounds in the intact GFP. Five TXA_2/PGH_2 receptor antagonists inhibited [^{3}H]U46619 binding in a concentration-dependent manner, and the potency series of the IC_{50} values was found to be SQ29548 ≥ ONO3708 > EP-045 > ONO11120 ≥ BM13177.

PGD_2 displaced the binding of [^{3}H]U46619 at concentrations greater than 1 μM, but complete suppression was not achieved at 100 μM (data not shown). PGE_1 and TXB_2, the stable metabolite of TXA_2, scarcely displaced the ligand binding. A similar displacement pattern was obtained in the crude membranes (data not shown). These results demonstrated that the [^{3}H]U46619 binding site in both rat GFP and membrane fractions represents the platelet TXA_2/PGH_2 receptor.

Inhibition of collagen-induced aggregation by TXA_2/PGH_2 *receptor antagonists.* As previously reported, TXA_2/PGH_2 may act as a trigger of the aggregation response of rat platelets stimulated by collagen [16]. As shown in Fig. 9, all of the receptor antagonists completely suppressed collagen-induced aggregation response. The potency for inhibition was found to be SQ29548 ≥ ONO3708 > EP-045 > ONO11120 ≥ BM13177. From log-log plots of K_i ([^{3}H]U46619 binding, Fig. 8) and IC_{50} (aggregation, Fig. 9) values, highly significant positive linear correlations were calculated for the inhibitory potencies of these antagonists between collagen-induced aggregation and [^{3}H]U46619 binding in intact GFP or crude membranes ($r = 0.995$, $P < 0.01$) (Fig. 10).

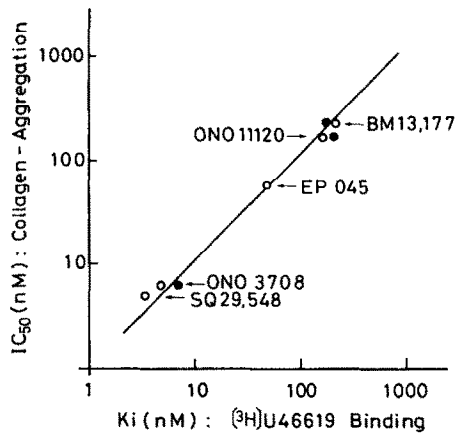

Fig. 10. Graphic correlation of receptor affinity and anti-aggregatory potency of several TXA_2/PGH_2 receptor antagonists. Open circles represent a log-log plot of K_i ([^{3}H]U46619 binding to intact GFP) versus IC_{50} (collagen-induced GFP aggregation) values employing data from Figs. 8 and 9 respectively. Closed circles represent a log-log plot of K_i ([^{3}H]U46619 binding to rat platelet membranes) versus IC_{50} (collagen-induced GFP aggregation) values. Each point represents the mean value of four experiments.

Table 1. Properties of inhibition of [^{3}H]U46619 binding to human platelet membranes and rat platelets by TXA_2/PGH_2 receptor antagonists

Antagonists	K_i (nM)	
	Human platelets*	Rat platelets†
SQ29548	8.5 ± 2.0	3.3 ± 0.8
ONO3708	9.2 ± 3.0	5.0 ± 1.2
EP-045	680 ± 85	51 ± 11
ONO11120	900 ± 80	170 ± 20
BM13177	1000 ± 120	200 ± 21

Data represent the mean values ± SE from four experiments.

* Human platelet membranes (0.45 mg) were incubated with 10 nM [^{3}H]U46619 and various concentrations of TXA_2/PGH_2 receptor antagonists in the presence of 20 mM $MgCl_2$ at 24° for 20 min. The K_i values were obtained by analysis of data similar to that for rat GFP as shown in Fig. 8.

† K_i values in rat GFP were determined by radioligand competition studies as described in the legend to Fig. 8.

Potencies of TXA_2/PGH_2 receptor antagonists for displacement of [^{3}H]*U46619 binding in human platelet membranes.* The specific binding of [^{3}H]U46619 to human platelet membranes was saturable and reversible, and was enhanced by addition of Mg^{2+} as was rat platelet membranes (data not shown). The Scatchard analysis of the specific [^{3}H]U46619 binding in the presence of 20 mM Mg^{2+} revealed the existence of a single type of binding site with a K_d of 125 nM and a B_{max} of 1640 fmol/mg protein (data not shown). This K_d value was comparable to that determined in intact human platelets [28, 30]. Five TXA_2/PGH_2 receptor antagonists completely suppressed [^{3}H]U46619 binding in a dose-dependent manner, and their K_i values are given in Table 1. The rank order of their abilities to inhibit [^{3}H]U46619 binding in human platelets paralleled with that in rat GFP (SQ29548 ≈ ONO3708 > EP-045 > ONO11120 ≈ BM13177). PGD_2 also displaced [^{3}H]U46619 binding to human platelet membranes at concentrations greater than 1 μM (data not shown), which corresponds with the results of intact human platelets using [^{125}I]PTA-OH as a radioligand [31].

DISCUSSION

Rat platelets have been regarded as unsuitable as a model for studying the action of prostaglandins in platelet activation [12], since the TXA_2 agonist can only evoke shape change and not aggregation [13, 15]. However, by addition of U46619 to rat GFP previously stimulated by collagen in the presence of indomethacin, complete aggregation was provoked as previously described [16, 17]. The ED_{50} value of U46619 for eliciting this response was estimated to be approximately 25 nM [16], which nearly corresponded to the K_d value (*ca.* 37 nM) for U46619 specific binding to rat GFP (Fig. 3). These data strongly suggest that the binding site of U46619 on rat GFP is the site responsible for eliciting platelet activation. The B_{max} value of U46619 was approximately 160 fmol/10^8 platelets, which was equivalent to *ca.* 1000 binding sites per platelet. This was about half of that reported for human platelets [28, 31], probably because rat platelets are smaller than human thrombocytes. All of the five TXA_2/PGH_2 receptor antagonists effectively displaced [^{3}H]U46619 binding to rat GFP (Fig. 8) as well as human platelet membranes. Good similarities were seen for the relative potencies of these antagonists for displacement of [^{3}H]U46619 binding between rat GFP and human membrane fraction (Table 1), suggesting that specificity of U46619 binding to the TXA_2/PGH_2 receptor on rat platelets may be almost the same as that on human platelets. The differences of K_i values for respective antagonists between rat and human platelets may be explained from the higher affinity of U46619 to rat (K_d = 37 nM) than human (K_d = 125 nM) platelets. Another correspondence between the TXA_2/PGH_2 receptors of rats and humans was that higher concentrations of PGD_2 effectively displaced [^{3}H]U46619 binding as shown in Fig. 8. This demonstrates a cross-reactivity of PGD_2 on the TXA_2/PGH_2 receptor which cancels out its antiaggregatory action in high concentrations as already reported for guinea pig platelets [32]. Kattleman and coworkers [28] provided evidence for the existence of a compartment, in which U46619 accumulated in a competitive but non-displaceable manner in human platelets. However, this non-displaceable pool was not observed in rat platelets, as [^{3}H]U46619 was completely displaced from its binding site by excess unlabeled U46619 (Fig. 2). These differences between rat and human platelets probably depend on the experimental procedures employed in each assay. Kattleman *et al.* [28] used washed platelets prepared by centrifugation and separated bound and free radioligands by centrifugation, while we employed the gel filtration method to prepare washed platelets [25], because it has several advantages such as good preservation of platelet morphology as well as physiological functions [33]. Another advantage of the rapid filtration method for separating bound/free ligands is that it may lead to a much lower level of non-specific binding.

We also succeeded in detecting specific binding activity for U46619 in the membrane fraction of rat and human platelets. Saussy *et al.* [26] obtained an extraordinary low value in human platelets for the inhibitory potency of U46619 against [^{125}I]PTA-OH binding to the membrane preparations compared with that in intact platelets. Alteration of binding affinity for the TXA_2/PGH_2 receptors in membrane preparations would occur probably due to the destruction of the integrities of the platelet membrane. We, therefore, examined the effects of some cations on the U46619 binding to the platelet membranes and found that the binding levels were increased significantly by addition of Mg^{2+} (Fig. 4). Scatchard analysis revealed increased affinity of binding sites with unaltered binding density in the presence of Mg^{2+}. These results strongly suggest that Mg^{2+} may increase the affinity of agonist for TXA_2/PGH_2 receptor in the platelet crude membranes. The effects of cations on the TXA_2/PGH_2 receptors in the platelet membrane serve as a contrast to the case of leukotriene D_4 and E_4 receptors in lung

membranes of the guinea pig [34, 35], the density of which was increased by Mg^{2+} as well as by other divalent cations. The K_d values, obtained from both Scatchard and kinetic analyses in the presence of Mg^{2+}, agreed well with the value from the intact GFP, suggesting that TXA_2/PGH_2 receptors on the membrane fractions in the presence of Mg^{2+} may have the same affinity as those on intact GFP. This was also supported by displacement data of several TXA_2/PGH_2 receptor antagonists having the same K_i values in both preparations (Fig. 10). As platelet membranes can be stored at $-70°$ without loss of binding affinity for over a month, a binding assay using crude membrane fractions can provide a direct means for evaluating synthetic TXA_2/PGH_2 receptor antagonists rather than intact platelets. Evidence for a good correlation between rat and human for the relative potencies of TXA_2/PGH_2 receptor antagonists (Table 1) also supports this idea.

Our recent studies clearly demonstrated an essential role of TXA_2/PGH_2 in mediating collagen to elicit platelet aggregation (Fig. 9). The IC_{50} values of several antagonists for inhibition of collagen-induced platelet aggregation were linearly correlated with K_i values for U46619 binding, as shown in Fig. 10. These findings not only support our proposed mechanism of TXA_2/PGH_2 action in collagen-stimulated platelets [16, 36], but they also offer a simple method for evaluating TXA_2/PGH_2 receptor antagonists.

More recently, we succeeded in characterizing the TXA_2/PGH_2 receptors in cultured smooth muscle cells (VSMC) of rat aorta by means of direct radioligand studies [37]. The rank order of several TXA_2/PGH_2 receptor antagonists for displacement of [^{3}H]-U46619 binding in rat platelets was almost comparable with their inhibitory potency for [^{3}H]SQ29548 binding in rat VSMC (SQ29548 > ONO3708 > ONO11120), suggesting that the TXA_2/PGH_2 receptors on rat platelets have specificity similar to that on vascular smooth muscles of the same species.

In conclusion, we emphasize that rat platelets may be used as a valuable tool for elucidating TXA_2 action on platelet aggregation as well as for screening TXA_2/PGH_2 receptor antagonists.

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REFERENCES

1. M. Hamberg, J. Svensson and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2994 (1975).
2. J. Svensson, M. Hamberg and B. Samuelsson, *Acta physiol. scand.* **98**, 285 (1976).
3. J. Svensson, K. Strandberg, T. Turemo and M. Hamberg, *Prostaglandins* **14**, 425 (1977).
4. R. A. Armstrong, R. L. Jones and N. H. Wilson, *Br. J. Pharmac.* **79**, 953 (1983).
5. B. J. R. Whittle and S. Moncada, *Br. med. Bull.* **39**, 232 (1983).
6. R. I. Levy, L. Wiener, P. Walinsky, A. M. Lefer, M. J. Silver and J. B. Smith, *Circulation* **61**, 1165 (1980).
7. M. L. Ogletree, *Fedn Proc.* **46**, 133 (1987).
8. A. M. Lefer and H. Darius, *Fedn Proc.* **46**, 144 (1987).
9. A. M. Lefer, *Drugs Today* **21**, 283 (1985).
10. D. E. Mais, P. J. Kochel, D. L. Saussy, Jr. and P. V. Halushka, *Molec. Pharmac.* **28**, 163 (1985).
11. D. E. Mais, R. M. Burch, D. L. Saussy, Jr., P. J. Kochel and P. V. Halushka, *J. Pharmac. exp. Ther.* **235**, 729 (1985).
12. Huzoor-Akbar and A. Khursheed, *Thromb. Res.* **41**, 555 (1986).
13. E. E. Nishizawa, D. J. Williams and C. L. Connell, *Thromb. Res.* **30**, 289 (1983).
14. D. E. MacIntyre, in *Platelets in Biology and Pathology II* (Ed. J. L. Gordon), pp. 211–47. Elsevier/North Holland, Amsterdam (1981).
15. T. Nakano, A. Terawaki and H. Arita, *J. Biochem., Tokyo* **99**, 1285 (1986).
16. K. Hanasaki, T. Nakano and H. Arita, *Thromb. Res.* **46**, 425 (1987).
17. T. Nakano, A. Terawaki and H. Arita, *J. Biochem., Tokyo* **101**, 1169 (1987).
18. H. Patscheke and K. Stegmeier, *Thromb. Res.* **33**, 277 (1984).
19. M. L. Ogletree, D. N. Harris, R. Greenberg, M. F. Haslanger and M. Nakane, *J. Pharmac. exp. Ther.* **234**, 435 (1985).
20. R. L. Jones, N. M. Wilson, R. A. Armstrong, V. Peesapati and G. M. Smith, *Leukotriene Res.* **11**, 351 (1983).
21. M. Katsura, T. Miyamoto, N. Hamanaka, K. Kondo, T. Terada, L. Ohgoki, A. Kawasaki and M. Tsuboshima, *Adv. Prostaglandin Thromboxane Leukotriene Res.* **11**, 351 (1983).
22. E. C. Witte, H. P. Wolff, M. Thiel, K. Stegmeier and E. Roesch, Jpn. Pat. Pub. No. 57–35910 (1982).
23. P. W. Sprague, J. E. Heikes, D. N. Harris and R. Greenberg, *Adv. Prostaglandin Thromboxane Leukotriene Res.* **11**, 337 (1983).
24. N. H. Wilson, V. Peesapati, R. L. Jones and K. Hamilton, *J. med. Chem.* **25**, 495 (1982).
25. P. N. Walsh, D. C. B. Mills and J. G. White, *Br. J. Haemat.* **36**, 281 (1977).
26. D. L. Saussy, D. E. Mais, R. M. Burch and P. V. Halushka, *J. biol. Chem.* **261**, 3025 (1986).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
28. E. J. Kattleman, D. J. Venton and G. C. Le Breton, *Thromb. Res.* **41**, 471 (1986).
29. J. A. Nelder and R. Mead, *Comput. J.* **2**, 308 (1965).
30. F. Ushikubi, M. Okuma, K. Kanaji, T. Sugiyama, T. Ogorochi, S. Narumiya and H. Uchino, *Thromb. Haemostas.* **57**, 158 (1987).
31. S. Narumiya, M. Okuma and F. Ushikubi, *Br. J. Pharmac.* **88**, 323 (1986).
32. S. Hamid-Bloomfield and B. J. R. Whittle, *Br. J. Pharmac.* **88**, 931 (1986).
33. O. Tangen, H. J. Berman and P. Marfey, *Thrombosis* **30**, 268 (1971).
34. H. M. Sarau, S. Mong, J. J. Foley, H-L. Wu and S. T. Crooke, *J. biol. Chem.* **262**, 4034 (1987).
35. S. Mong, M. O. Scott, M. A. Lewis, H-L. Wu, G. K. Hogaboom, M. A. Clark and S. T. Crooke, *Eur. J. Pharmac.* **109**, 183 (1985).
36. K. Hanasaki, T. Nakano and H. Arita, *J. Biochem., Tokyo.* **102**, 5 (1987).
37. K. Hanasaki, K. Nakano, H. Kasai, H. Arita, K. Ohtani and M. Doteuchi, *Biochem. biophys. Res. Commun.* **150**, 1170 (1988).