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CHARACTERIZATION OF PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR BY A NOVEL THROMBOXANE RECEPTOR ANTAGONIST, [^{3}H]S-145

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Abstract—The specific binding sites for S-145, a novel thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptor antagonist with weak partial agonistic activity, were studied in human platelet membranes. [^{3}H]S-145 displayed high affinity and specificity, as well as saturable and displaceable binding, to a single class of recognition sites with the same maximum number of sites (2100 fmol/mg protein) as the other two TXA_2/PGH_2 receptor antagonists, [^{3}H]SQ29,548 and [^{3}H]ONO3708. Binding of S-145 to the platelet membranes was enhanced by divalent cations (Mg^{2+} and Ca^{2+}), and the binding affinity in the presence of 20 mM $MgCl_2$ was 0.75 nM, a value which was smaller than those of SQ29,548 (8.7 nM) and ONO3708 (3.7 nM). The rank order of potency (K_i) for a series of TXA_2/PGH_2 receptor antagonists to displace [^{3}H]S-145 binding to the membranes was correlated with those determined from [^{3}H]SQ29,548 or [^{3}H]ONO3708 binding to the same preparations. Kinetic analysis for the binding of the above radiolabeled antagonist to the crude platelet membranes revealed that the dissociation rate constant (K_{-1}) for S-145 was much smaller than that for other ligands in human, rat and rabbit platelets. The extremely slow dissociation of S-145 from the receptors may explain the long-lasting characteristic of this compound *in vivo* as well as the abolishment of partial agonistic activity.

The metabolism of arachidonic acid by platelets results in the formation of thromboxane A_2 (TXA_2),† which exerts potent biological actions [1, 2]. Because of its potent proaggregatory and vasoconstrictor effects, it is believed to augment hemostasis and also to contribute to the pathogenesis of a variety of vascular disorders due to the promotion of ischemic damage by constricting coronary arteries and the formation of platelet aggregates and microthrombi [3, 4]. Therefore, many efforts have been made to develop specific inhibitors of TXA_2 synthesis as well as thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptor antagonists [5, 6], which have provided the tools for investigations into the mechanisms of TXA_2 action. As for biochemical characterization of TXA_2/PGH_2 receptors, various radiolabeled analogues of TXA_2 have been synthesized, such as [^{3}H]13-azaprostanoic acid [7], [^{3}H]U46609 [8], [^{3}H]U46619 [9–11], [^{125}I]PTA-OH [12–16], [^{125}I]-*p*-OH-SQ28,668 [17] and [^{3}H]SQ29,548 [18]. These radioligands provided varying degrees of success in binding studies for the characterization of the TXA_2/PGH_2 receptors in the platelets. More recently, we succeeded in identifying rat TXA_2/PGH_2 receptors in platelets [19], vascular smooth muscle cells [20] and vascular endothelial cells [21], and also compared their ligand binding specificities as well as receptor numbers per cell [21].

Our recent studies have shown that one of the synthetic compounds in our laboratories, S-145 [22], is a powerful and selective TXA_2/PGH_2 receptor antagonist on platelets and vascular smooth muscle preparations [23].‡ S-145 has specifically high potency without any species differences in the affinity for TXA_2/PGH_2 receptors among rat, rabbit and human platelets [23]. Furthermore, S-145 is an interesting tool for studying the signal transduction of platelet shape change [24], since this ligand provokes transient and reversible shape change, but no aggregation response in the platelets [23]. To obtain more information on the receptor binding of S-145, we synthesized tritium-labeled S-145 ([^{3}H]S-145) and examined it for its binding characteristics by kinetic analysis of the platelet membrane fractions.

In the present paper, we identified S-145 as the most potent and possibly long-lasting antagonist for the platelet thromboxane receptor thus far available.

MATERIALS AND METHODS

Materials

[^{3}H]SQ29,548 (40.0 Ci/mmol) and [^{3}H]U46619 (22.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Collagen (type IV, soluble), U46619, PGE_1, PGD_2, $PGF_{2\alpha}$ and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO). TXB_2 was purchased from Funakoshi (Osaka, Japan). PMSF and EDTA were from Nakarai Chemicals (Kyoto, Japan). S-145 [(±)-5(Z)7-[3-*endo*-phenylsulfonylamino[2.2.1]bicyclo-

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† Abbreviations: TXA_2 (B_2), thromboxane A_2 (B_2); PGH_2 (E_1, D_2, $F_{2\alpha}$), prostaglandin H_2 (E_1, D_2, $F_{2\alpha}$); PRP, platelet-rich plasma; GFP, gel-filtered platelets; PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and RI, radioisotope.

‡ Nakajima M, Otani K, Doteuchi M and Ueda M, Pharmacological studies of a novel thromboxane A_2 receptor antagonist, S-145. 1. Effects of S-145 on isolated rat, cat and monkey arteries. Taipei Conference on Prostaglandin and Leukotriene Research, p. 173, 1988.

hept-2-*exo*-yl]heptenoic acid] as well as its (+)- or (−)-isomer [22], SQ29,548 (1S-[1α,2β(5Z),3β,4α]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) [25], 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) [26], ONO11120 (9,11-dimethylmethano-11,12-methano-16-phenyl-13,14-dihydro-13-aza-15αβ-ω-tetranor-TXA_2) [27] and EP-045 [(±)-5-*endo*-(6′-carboxyhex-2′Z-enyl-6-*exo*[*N*-(phenylcarbamoyl)hydrazono-methyl]-bicyclo[2.2.1]heptane)] [28] were synthesized in the Shionogi Research Laboratories, Osaka. A 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 the binding study 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 0.9% NaCl before use.

Chemical preparation of [^{3}H]S-145 *and* [^{3}H]ONO3708

(1) (±)-5(Z)-7-(*endo*-3-[4-^{3}H]Benzenesulfonyl-aminobicyclo[2.2.1]hept-*exo*-2-yl)hept-5-enoic acid sodium salt, [^{3}H]S-145 sodium salt, was prepared as follows. To a stirred solution of methyl (±)-5(Z)-7-*endo*-3-aminobicyclo[2.2.1]hept-*exo*-2-yl)hept-5-enoate (48 mg, 0.2 mmol) in anhydrous benzene (2.5 ml) were added triethylamine (30 mg, 0.3 mmol) and a solution of [4-^{3}H]benzene-sulfonyl chloride (98 mCi, 0.708 mg, 0.004 mmol, 24.5 Ci/mmol, Amersham) in benzene (25 ml). The mixture was concentrated *in vacuo* (60 mm Hg) at 35° to about 2 ml and stirred for 2.0 hr at room temperature. The reaction mixture was evaporated *in vacuo* below 50°, leaving a viscous oil which was purified by column chromatography on silica gel (Merck No. 7734, 200 mg; elution with benzene–ethyl acetate 2:1), giving pure methyl (±)-(Z)-7-(*endo*-3-[4-^{3}H]benzenesulfonylaminobicyclo-[2.2.1]hept-*exo*-2-yl)hept-5-enoate ([^{3}H]S-145 methyl ester) (45 mCi, 0.00184 mmol) as a viscous oil. [^{3}H]S-145 methyl ester was converted into [^{3}H]S-145 sodium salt by saponification with 1 N sodium hydroxide (0.1 ml) in methanol (0.5 ml) with stirring for 5 hr at 45°. The mixture was then evaporated *in vacuo* below 25°, giving crude [^{3}H]S-145 sodium salt as a crystalline residue which was purified by chromatography (SEP-$PAK_{C\text{-}18}$; elution with 20% aqueous ethanol). The fractions containing the almost pure target compound were combined and evaporated *in vacuo* to dryness, giving [^{3}H]S-145 sodium salt (41.7 mCi, 0.64 mg, 24.5 mCi/mmol, radiochemical purity 99.1%) in 42.5% overall radiochemical yield based on [^{3}H]benzenesulfonyl chloride. The sodium salt of [^{3}H]S-145 was dissolved in 95% ethanol, and the RI concentration was adjusted to 500 μCi/ml. Radiochemical purity was measured by TLC autoradiogram followed by liquid scintillation counting and HPLC ($Nucleosil_{C\text{-}18}$, 4.6 mm × 15 cm; mobile phase, CH_3CN:MeOH:H_2O:AcOH = 300:200:300:1; detection, UV 220 nm and radioactivity measured with a Packard Trace II-7150 detector). The product obtained was identified with authentic unlabeled compound by comparison of TLC (R_f) and HPLC (retention time).

(2) 5-(Z)-7-(1*S*,2*S*,3*S*,5*R*,2′*R*)-(3-([2′-^{3}H]-2′-Cyclopentyl-2′-hydroxyacetylamino)-6,6-dimethyl-bicyclo[3.1.1]hept-2-yl)-hept-5-enoic acid sodium salt, [^{3}H]ONO3708 sodium salt, was prepared as follows. To a stirred solution of methyl 5-(Z)-7-(1*S*,2*S*,3*S*,5*R*)-(3-(2′-cyclopentyl-2′-oxo-acetamino)-6,6-dimethylbicyclo[3.1.1]hept-2-yl)hept-5-enoate (15 mg, 0.0372 mmol, prepared from ONO3708 methyl ester by oxidation) in ethanol (0.3 ml) was added sodium borohydride[^{3}H] (500 mCi, 0.275 mg, 0.00725 mmol, Dupont/NEN Research Product). After this was stirred for 4.0 hr at 0° and for 1.5 hr at room temperature, 10% aqueous acetic acid (0.1 ml) and water (2.5 ml) were added to the solution and the reaction solution was extracted with ether (2.5 ml × 3). The extracts were washed with 5% sodium bicarbonate and water, dried over sodium sulfate and evaporated *in vacuo*, leaving an oily residue (*ca.* 15 mg). After removing labile tritium by repeated co-evaporation with benzene–methanol (4:1, 2.5 ml) and 95% ethanol (2 ml × 3), the desired [^{3}H]ONO3708 methyl ester was isolated by preparative TLC (Merck KG precoated plate No. 5715; solvent system, benzene–ethyl acetate 4:1). The [^{3}H]ONO3708 methyl ester obtained (81.5 mCi, 3.14 mg, 0.00776 mmol, 10.5 Ci/mmol radiochemical purity 98.9%) was converted into [^{3}H]ONO3708 sodium salt by saponification with 1 N sodium hydroxide (0.2 ml) in ethanol (0.5 ml) with stirring for 1 hr at room temperature. Purification and identification were carried out in a manner similar to that described for the preparation of [^{3}H]S-145 sodium salt, giving [^{3}H]ONO3708 sodium salt (67.5 mCi, 0.0065 mmol, 10.3 Ci/mmol, radiochemical purity 99.4%) as a 514 μCi/ml ethanol solution.

Preparation of gel-filtered platelets (GFP) and platelet membranes

Human blood was drawn by venipuncture from normal volunteers, who had not taken any medication for at least 14 days, 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. Blood was collected from rat and rabbit as described previously [23]. Platelet-rich plasma (PRP) was obtained by centrifugation at 160 *g* for 10 min. GFP and platelet membranes were prepared from PRP as described in the previous paper [19]. GFP were suspended in the 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% BSA, pH 7.35) to a final concentration of 5×10^8 cells/ml for rat and rabbit platelets or 3×10^8 cells/ml for human platelets. The platelet membranes were suspended in the incubation buffer (50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 10 μM indomethacin and 0.3 mM PMSF) to a final protein concentration of about 5 mg/ml and stored at −70° until use. Protein concentration was measured by the method of Lowry *et al.* [29] with

human serum albumin as the standard.

Binding assays

The binding assays in GFP and membrane fractions were carried out according to the method used in the [^{3}H]U46619 binding as described previously [19]. Briefly, the binding studies were performed by incubating GFP (3.4×10^8 cells) or platelet membranes (0.1 to 0.4 mg) with each ^{3}H-labeled TXA_2/PGH_2 receptor ligand in a total volume of 0.4 ml in siliconized glass tubes at 24°. After the incubation, the 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. The filter was then washed four times with ice-cold 0.9% NaCl, and analyzed for contents of radioactivity using a scintillation counter (Aloka, LSC-700). All filtration procedures were completed within 10 sec. Binding data are presented as the specific binding which is determined as the differences between the amount of [^{3}H]radioligands bound in the presence and absence of a 10 μM concentration of the respective unlabeled antagonists. The specific binding of [^{3}H]S-145 (2.5 nM) was linearly dependent upon the platelet membrane protein concentration from 0.05 to 1.0 mg/ml. The specific binding of [^{3}H]S-145 (2.5 nM), [^{3}H]SQ29,548 (4.1 nM) and [^{3}H]ONO3708 (7.5 nM) to 0.1 mg of human platelet membranes represented, respectively, 98 ± 1, 97 ± 2 and 96 ± 3% of the total binding.

Measurement of the platelet shape change and aggregation

Rat GFP (5×10^8 cells/ml) or human PRP (2×10^8) were preincubated, respectively, with or without 1 mM $CaCl_2$ for 2 min at 37°, and then various stimuli were added. The aggregation and shape change were monitored simultaneously with an aggregometer (model PAT-6A, Niko Bioscience Co. Ltd) in terms of the increase and decrease in light transmission respectively.

Statistical analysis

Linear regression analysis of the binding data was performed according to the standard methods [30].

RESULTS

Effects of cations on [^{3}H]S-145 *binding to human platelet membranes*

When [^{3}H]S-145 (2.5 nM) was incubated at 24° with human platelet membranes suspended in the incubation buffer containing a final concentration of 4.5 mM EDTA, [^{3}H]S-145 binding to the membranes reached a stable, steady-state level within 60 min and remained at the same level for up to 180 min. The effects of various cations on [^{3}H]S-145 specific binding to the membranes were determined and are shown in Fig. 1. In the concentration range of 20–100 mM, Ca^{2+} and Mg^{2+} enhanced [^{3}H]S-145 binding by about 2-fold, whereas monovalent cations (Na^+ and K^+) did not affect significantly the bindings at concentrations up to 100 mM. Our previous studies showed the Mg^{2+}-induced enhancement of the agonist binding affinity for the TXA_2/PGH_2 receptors in

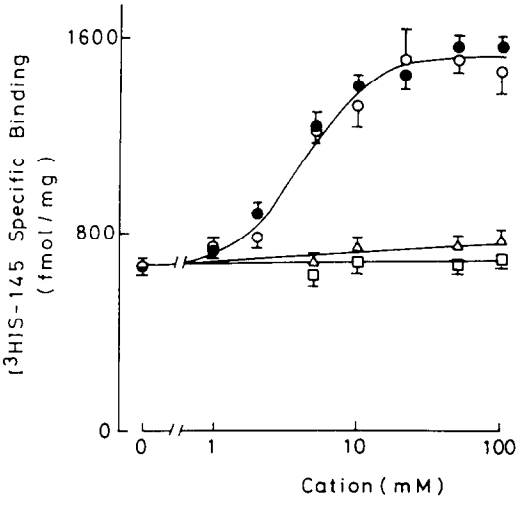

Fig. 1. Effects of cations on specific binding of [^{3}H]S-145 to human platelet membranes. Human platelet crude membranes (0.1 mg protein), suspended in the incubation buffer containing 4.5 mM EDTA (final concentration), were incubated with 2.5 nM [^{3}H]S-145 at 24° for 90 min in the presence of various concentrations of $MgCl_2$ (○), $CaCl_2$ (●), NaCl (□) and KCl (△). Means ± SE, calculated from the average of three separate experiments, are shown.

the membrane fractions [19]. Scatchard analysis for the [^{3}H]S-145 binding in the absence or presence of 20 mM $MgCl_2$ revealed increased affinity of binding sites without any changes of binding density in the presence of Mg^{2+}. Ca^{2+} was found to have the same effect (data not shown). Based on these results, all the following binding studies using platelet membranes were performed in the presence of 20 mM $MgCl_2$.

Equilibrium binding studies of [^{3}H]S-145, [^{3}H]SQ29,548 *and* [^{3}H]ONO3708

To characterize the [^{3}H]S-145 binding, we also used radiolabeled SQ29,548 and ONO3708, both of which are known as potent and selective TXA_2/PGH_2 receptor antagonists in the platelets [9]. Studies of saturation binding of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 to human platelet membranes revealed that non-specific binding increased linearly with rising concentrations of each [^{3}H]radioligand, whereas the specific binding exhibited complete saturability. As shown in Fig. 2, Scatchard analysis of these data indicated the existence of a single class of binding sites for each TXA_2/PGH_2 receptor antagonist in the membrane fractions of human platelets. The binding affinities (K_d) for [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 were, respectively, 0.75 ± 0.25, 8.7 ± 2.1 and 3.7 ± 1.4 nM. The maximum number of binding sites (B_{max}) was found to be almost the same among the three TXA_2/PGH_2 receptor antagonists (2100 ± 80 fmol/mg protein). From a comparison with their K_d values, the rank order of binding affinity for human platelet membranes was found to be S-145 > ONO3708 > SQ29,548. The K_d value of [^{3}H]S-145 binding to membrane fractions in the pres-

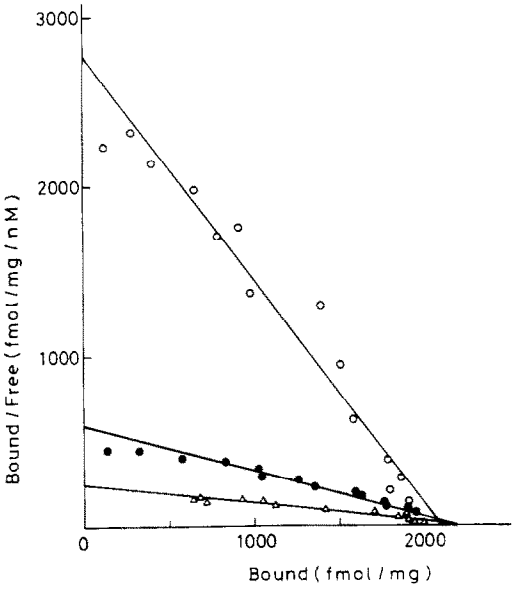

Fig. 2. Scatchard analysis of the binding of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to human platelet membranes. Human platelet membranes (0.1 mg protein) were incubated with increasing concentrations of [^{3}H]S-145 (○), [^{3}H]SQ29,548 (△) or [^{3}H]ONO3708 (●) in the presence of 20 mM $MgCl_2$ at 24° for 120, 30 and 30 min respectively. Non-specific binding in the presence of 10 μM antagonist was subtracted from each point. Each point is the mean value of triplicate determinations for three experiments.

ence of 20 mM $MgCl_2$ was almost the same as the value determined from intact gel-filtered human platelets (0.66 nM).

Specificity of [^{3}H]S-145 *binding to human platelet membranes*

A number of structurally dissimilar compounds were examined for their abilities to compete with the binding of [^{3}H]S-145 (0.5 nM) to human platelet membranes. As shown in Fig. 3, four well-known TXA_2/PGH_2 receptor antagonists and S-145, as well as its isomers, suppressed [^{3}H]S-145 binding in a concentration-dependent manner, and the potency series of the IC_{50} values was found to be (+)-isomer > S-145 > ONO3708 > SQ29,548 > (−)-isomer > EP-045 ≈ ONO11120. In addition, U46619, a TXA_2 mimetic agonist, also completely blocked the binding. PGD_2 caused displacement at concentrations greater than 1 μM, but complete suppression was not observed even at 100 μM. PGE_1, $PGF_{2\alpha}$ and TXB_2 scarcely caused displacement of the ligand binding. These results demonstrate that [^{3}H]S-145 bound to the platelet TXA_2/PGH_2 receptor with stereoselectivity and specificity.

Competitive inhibition of [^{3}H]S-145 *binding by* U46619

Human platelet membranes were incubated with increasing concentrations of [^{3}H]S-145 (0.23 to 2.1 nM) in the absence and the presence of three concentrations of U46619 (0.1, 0.3 and 1.0 μM) (Fig. 4). The K_d values, calculated from the linear regression analysis of the data by Lineweaver–Burk plot, were 0.80 nM in the absence of U46619 and 1.1, 1.4 and 2.7 nM in the presence of 0.1, 0.3 and 1.0 μM U46619 respectively. Scatchard analysis confirmed that B_{max} was not modified by these concentrations of U46619; however, the K_d values increased to 0.91, 1.6 and 2.6 nM, respectively, from the control value of 0.82 nM. These data indicate that U46619 inhibited the [^{3}H]S-145 specific binding in a competitive manner.

Comparison of the inhibitory potencies of TXA_2/PGH_2 *receptor antagonists against* [^{3}H]S-145, [^{3}H]U46619, [^{3}H]SQ29,548 *and* [^{3}H]ONO3708 *binding*

To confirm the binding specificity of [^{3}H]S-145 towards human platelets, the displacement of other ^{3}H-labeled TXA_2/PGH_2 receptor ligands by the above seven antagonists (Fig. 3) was performed. All the seven TXA_2/PGH_2 receptor antagonists blocked the bindings of [^{3}H]U46619, [^{3}H]SQ29,548 and [^{3}H]ONO3708 to human platelet membranes in a concentration-dependent manner. Using the IC_{50} values and the K_d values for the respective [^{3}H]radioligands derived from Scatchard analysis (from Fig. 2 or [^{3}H]U46619 from Ref. 23), K_i values for the receptor antagonists were approximated according to the Cheng–Prusoff equations [31]. The negative logarithms of K_i values for seven TXA_2/PGH_2 receptor antagonists against [^{3}H]S-145 binding were highly correlated to the corresponding K_i values against [^{3}H]U46619 (Fig. 5A, $r = 0.97$), [^{3}H]SQ29,548 (Fig. 5B, $r = 0.99$) or [^{3}H]ONO3708 (Fig. 5C, $r = 0.98$). Furthermore, the K_i values of unlabeled S-145, SQ29,548 and ONO3708 were almost comparable to the respective K_d values obtained from saturation studies (Fig. 2). Thus, among the compounds studied, S-145 appears to be the most potent antagonist against the TXA_2/PGH_2 receptors in human platelets.

Kinetic characteristics of [^{3}H]S-145, [^{3}H]SQ29,548 *or* [^{3}H]ONO3708 *binding to human platelet membranes*

For further characterization of the high affinity of [^{3}H]S-145 for the TXA_2/PGH_2 receptors in human platelet membranes, kinetic analysis for the receptor binding was performed and compared with the bindings of [^{3}H]SQ29,548 and [^{3}H]ONO3708. Figure 6 shows the association time–course of various concentrations of respective [^{3}H]radioligands. The association rate increased with increasing concentrations of the respective receptor antagonists. The observed rate constant (K_{obs}) was determined from the slope of the pseudo first-order rate plot for the time–course of each binding. K_{obs} was, then, plotted versus the corresponding ligand concentration [L] (Fig. 7), and the slope of the plot revealed the true rate constant of association (K_1) for each receptor antagonist (shown in Table 1). The K_{obs} values seem to be in direct proportion to the [^{3}H]S-145 concentrations as compared with [^{3}H]SQ29,548 and [^{3}H]ONO3708. To determine the dissociation rate constant (K_{-1}), the membranes were incubated with each ^{3}H-labeled TXA_2/PGH_2 receptor antagonist to ensure that equilibrium had been reached.

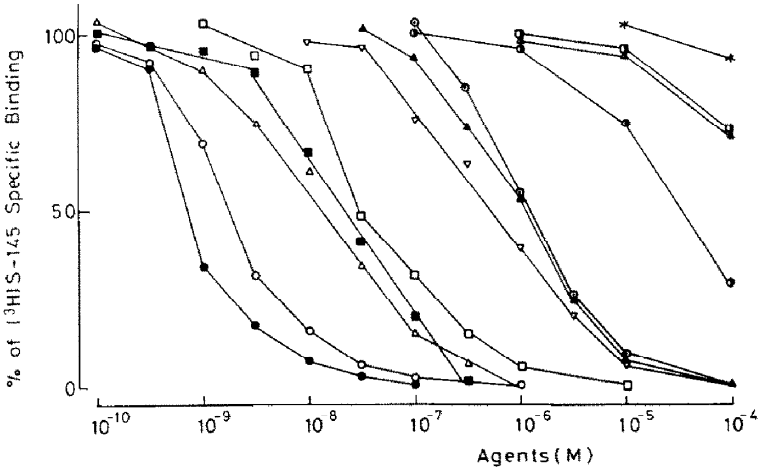

Fig. 3. Displacement of specific [^{3}H]S-145 binding to human platelet membranes by S-145 and related compounds. Human platelet membranes (0.1 mg protein) were incubated with 0.5 nM [^{3}H]S-145 in the presence of various concentrations of S-145 (○), (+)-isomer (●), (−)-isomer (□), SQ29,548 (■), ONO3708 (△), ONO11120 (▲), EP-045 (⊙), U46619 (▽), PGD_2 (◑), PGE_1 (◭), $PGF_{2\alpha}$ (◨) and TXB_2 (*). Incubation was carried out at 24° for 120 min. The specific binding was found by subtracting non-specific binding obtained with 10 μM S-145 from each point. The control value (100%) was defined as the specific binding of [^{3}H]S-145 in the absence of these compounds. Each point is the average of results from three independent experimental determinations performed in triplicate.

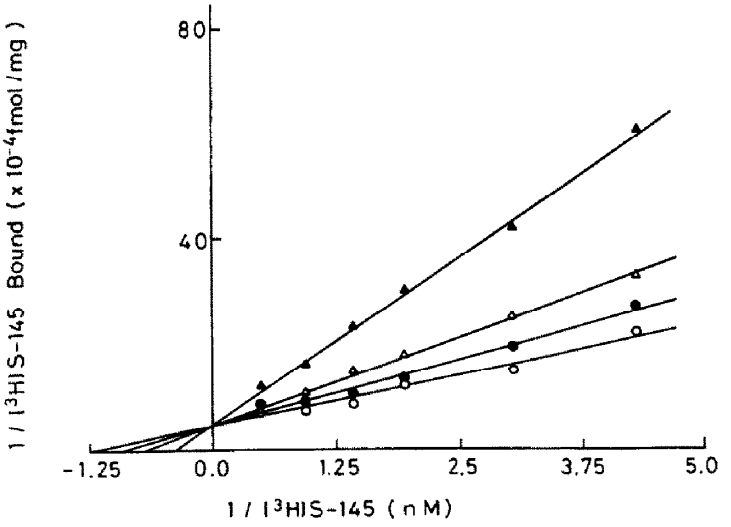

Fig. 4. Lineweaver–Burk representation of competitive inhibition of [^{3}H]S-145 binding by U46619. Human platelet membranes (0.1 mg protein) were incubated with increasing concentrations of [^{3}H]S-145 (from 0.23 to 2.1 nM) in the absence (○) or the presence of 0.1 μM (●), 0.3 μM (△) and 1.0 μM (▲) U46619 at 24° for 120 min. The fitting of the results with a least-squares linear regression analysis allowed the calculation of K_d values. Each point is the mean of triplicate determinations.

At an arbitrary zero time point, excess unlabeled antagonist (10 μM) was added, and the time course of displacement of each [^{3}H]radioligand from its binding sites was determined. As shown in Fig. 8, the dissociation of [^{3}H]SQ29,548 and [^{3}H]ONO3708 from specific binding sites was rapid and completed within 90 min, whereas that of [^{3}H]S-145 was much slower, and the time ($T_{1/2}$), at which one-half of its equilibrium level had been reached, was about 240 min. Linear transformation of these data (Fig. 8, insert) revealed a K_{-1} for the binding of each receptor antagonist to human platelet membranes, as shown in Table 1.

Species differences in the kinetic characteristics of [^{3}H]S-145 *and* [^{3}H]ONO3708 *bindings to the platelet membranes*

The same kinetic analysis of the binding studies for [^{3}H]S-145 and [^{3}H]ONO3708 was carried out in the platelet membranes of rat and rabbit. The K_1

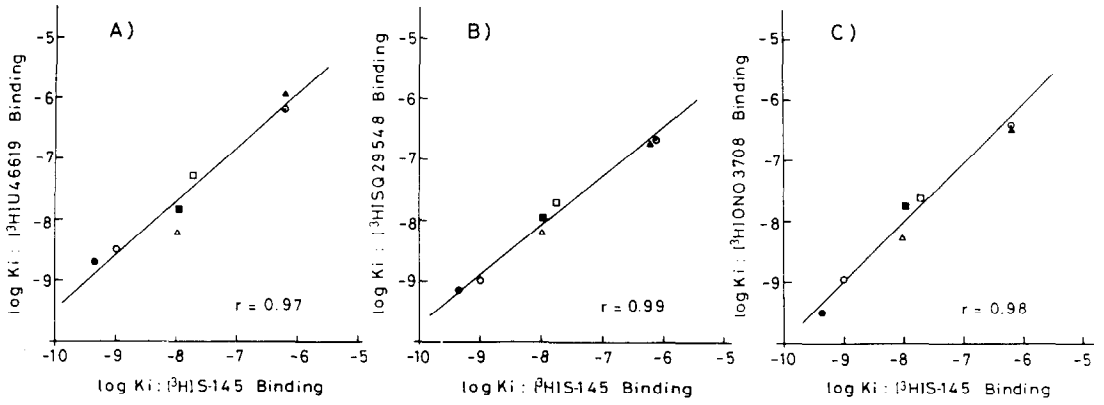

Fig. 5. Correlation between the potencies of several TXA_2/PGH_2 receptor antagonists to displace [^{3}H]S-145 binding and to inhibit specific binding of [^{3}H]U46619 (A), [^{3}H]SQ29,548 (B) and [^{3}H]ONO3708 (C) in human platelet membranes. The IC_{50} values for S-145 (○), (+)-isomer (●), (−)-isomer (□), SQ29,548 (●), ONO3708 (△), ONO11120 (▲) and EP-045 (⊙) were determined, respectively, from the concentration–inhibition curves for the binding of 0.5 nM [^{3}H]S-145 (Fig. 3), 12.4 nM [^{3}H]U46619 (0.38 mg protein at 24° for 30 min), 3.3 nM [^{3}H]SQ29,548 (0.10 mg protein at 24° for 30 min) or 7.0 nM [^{3}H]ONO3708 (0.12 mg protein at 24° for 90 min). The K_i values were calculated from the Cheng–Prusoff equation. Each point is the mean value of three experiments performed in triplicate. The reported points were fitted by least-squares linear regression.

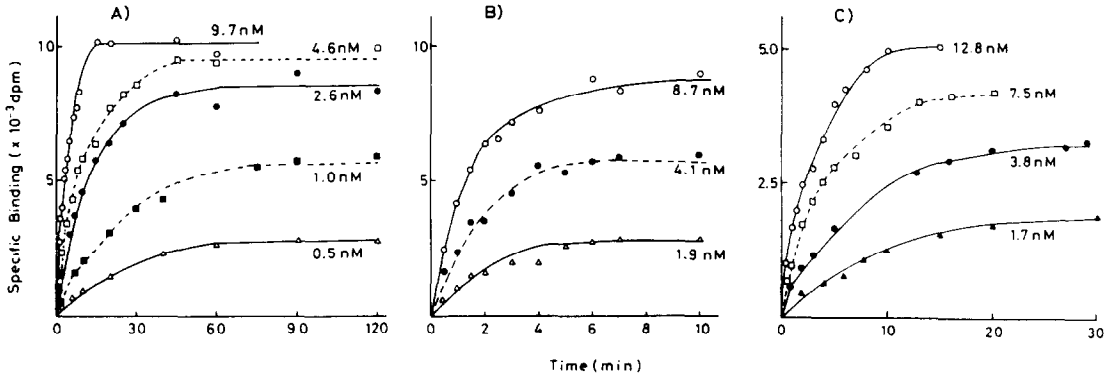

Fig. 6. Time–course of association of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 to the membrane preparations of human platelets. Human platelet membranes (0.12 mg protein) were incubated with various concentrations of [^{3}H]S-145 (A), [^{3}H]SQ29,548 (B) or [^{3}H]ONO3708 (C) in the presence of 20 mM $MgCl_2$ at 24°. The samples were withdrawn at the indicated times. Each point is the mean value of triplicate determinations.

and K_{-1} values for the binding of each antagonist are summarized in Table 1. As for [^{3}H]S-145 binding, there were no large differences in either rate constant among the three species. In contrast, specific binding of [^{3}H]ONO3708 was not significantly detected in rabbit platelet membranes, which agrees well with previous reports that rabbit platelets showed no significant binding for ^{125}I-labeled pinane thromboxane antagonist (PTA-OH) [14]. On the other hand, [^{3}H]ONO3708 binding to rat platelets was found to have kinetic parameters similar to those of human platelets. The most obvious feature in the [^{3}H]S-145 binding to the platelet TXA_2/PGH_2 receptors is the extremely small dissociation rate constant.

Long duration of the antagonistic activity of S-145 *in platelet* TXA_2/PGH_2 *receptors*

Owing to the extremely slow dissociation of S-145 from the platelet TXA_2/PGH_2 receptors, S-145 was expected to block the receptors for a long time. To assess this possibility, we used rat platelets, because [^{3}H]U46619 binding activity and collagen-induced aggregation in rat platelets are directly dependent on the action of TXA_2/PGH_2 [19, 32]. As shown in Table 2, pretreatment of rat washed-platelets with 1 μM S-145 for 10 min, followed by washing of the cells by the gel filtration method, resulted in *ca.* a 44% decrease, compared to the control cells, in the activity for the above two responses, whereas aggregation provoked by 0.4 units/ml thrombin was unaffected. However, no change was observed in the above responses of platelets pretreated with 1 μM ONO3708 (Table 1) or SQ29,548 (data not shown), indicating that S-145 had a longer antagonistic action against the platelet TXA_2/PGH_2 receptors than ONO3708 or SQ29,548.

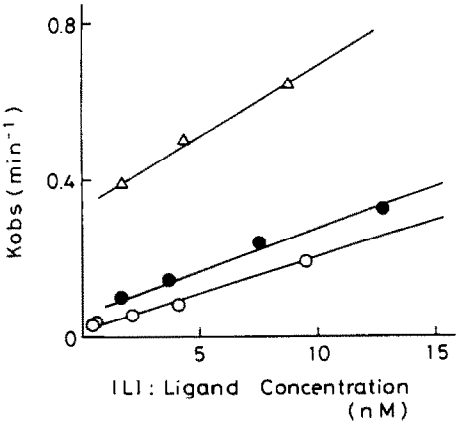

Fig. 7. Association rate of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 binding to human platelet membranes. The data, obtained from the association time-course of the binding for [^{3}H]S-145 (○), [^{3}H]SQ29,548 (△) and [^{3}H]ONO3708 (●) (Fig. 6), were analyzed assuming a pseudo-first-order process. Then the slopes (K_{obs}) were plotted as a function of the corresponding ligand concentration [L]. The slope of the plot is the rate constant of association (K_1).

Inhibition of U46619*-induced aggregation of human platelets by* S-145

The effect of S-145 on U46619-induced platelet aggregation was compared with that of SQ29,548. Antagonism by SQ29,548 against the aggregation provoked by U46619 is shown in Fig. 9A. Increasing concentrations of SQ29,548 caused a concentration-related parallel shift to the right of the concentration–response relationships for U46619. Analysis of Schild plot [33] gave a pA_2 value of 8.0 with a slope of −0.94, not significantly different from −1.0, indicating that SQ29,548 is a competitive receptor antagonist [34]. The platelet aggregation was also inhibited by pretreatment with S-145 (5–50 nM) in a concentration-dependent manner (Fig. 9B). However, no pA_2 value was determined, as pretreatment with increasing concentrations of S-145 diminished the maximal achievable aggregation response. Thus, S-145 suppressed the U46619-evoked aggregation response in an apparently non-competitive manner.

Abolishment of the S-145*-induced agonistic action*

Although S-145 is a potent TXA_2/PGH_2 receptor antagonist in the platelets, this ligand also possesses a partial agonistic activity [23]. As shown in Fig. 10A, 1 μM S-145 provoked a transient and reversible shape change without aggregation and secretion in human platelets, whereas U46619 evokes full aggregation [35], as we have previously reported in rabbit platelets [24]. However, pretreatment of the platelets six times with a low concentration of S-145 (2×10^{-8} M), which did not elicit platelet shape change, caused suppression of the platelet shape change induced by 1 μM S-145 (Fig. 10B). By increasing the number of times of pretreatment with the low concentration of S-145, the partial agonism induced by 1 μM S-145 could be proportionally diminished (Fig. 10C). The gradual increment of S-145 from lower (1 nM) to higher (1 μM) concentrations also abolished the S-145-induced partial agonistic response (data not shown).

DISCUSSION

The present study describes the binding characteristics of S-145, a novel TXA_2/PGH_2 receptor antagonist, to the platelets. [^{3}H]S-145 displayed high affinity and specificity, as well as saturable and displaceable binding in human platelets. The binding affinity of [^{3}H]S-145 for the platelet membrane preparations was increased by addition of Mg^{2+} with unaltered binding density (Fig. 1). The effects of Mg^{2+} were equivalent to the [^{3}H]U46619 binding to the TXA_2/PGH_2 receptors in the membrane preparations of rat, rabbit and human platelets [19, 23]. The similarity of K_d values for S-145 determined in intact platelets and membrane preparations in the presence of 20 mM $MgCl_2$ lent further support to our previous observation that TXA_2/PGH_2 receptors of intact platelets as well as platelet membrane preparations have the same affinity in the presence of Mg^{2+} [19]. The requirement of Mg^{2+} for the optimum binding of the ligand was also reported in the purified PGE_1 receptors from human platelets [36]. Ca^{2+} had the same effect on the [^{3}H]S-145 binding to human

Table 1. Parameters describing the binding of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to rat, rabbit and human platelet membranes

Ligand	Species	K_1 ($M^{-1}\,min^{-1}$)	K_{-1} (min^{-1})	$K_d = K_{-1}K_1$ (nM)
[^{3}H]S-145	Rat	1.8×10^7	0.006	0.34
	Rabbit	3.3×10^7	0.011	0.33
	Human	1.9×10^7	0.003	0.16
[^{3}H]ONO3708	Rat	2.9×10^7	0.047	1.6
	Rabbit	ND*	ND	ND
	Human	2.5×10^7	0.046	1.9
[^{3}H]SQ29,548	Human	3.4×10^7	0.26	7.6

The studies on rat and rabbit platelet membranes were performed and the data analyzed as described for human platelets (Figs 7 and 8). The results shown are from at least two experiments using triplicate determinations.

* Not detected.

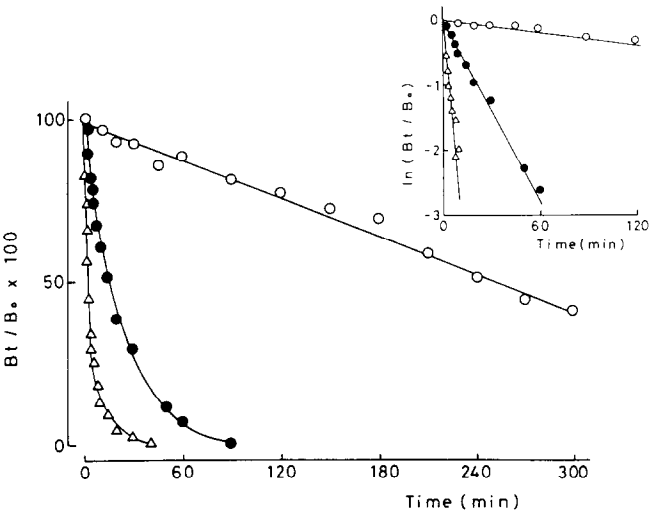

Fig. 8. Time–course of dissociation of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 from human platelet membranes. Association of human platelet membranes (0.12 mg protein) with 6.4 nM [^{3}H]S-145 (○), 5.5 nM [^{3}H]SQ29,548 (△) or 9.8 nM [^{3}H]ONO3708 (●) was carried out at 24° for 60, 30 and 30 min respectively. Next, a 10 μM concentration of each unlabeled receptor antagonist was added at time zero to initiate displacement of ^{3}H-labeled TXA_2/PGH_2 receptor antagonist from its specific binding site. The data were expressed with the value of the specific binding at time zero at 100%. B_t is the ^{3}H-labeled antagonists specifically bound at any particular time t and B_0 is the amount specifically bound at time zero. Each point is 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}.

Table 2. Effect of S-145 pretreatment on the aggregation and [^{3}H]U46619 binding activity in rat platelets

Pretreatment	Aggregation (%) Collagen	Aggregation (%) Thrombin	[^{3}H]U46619 binding (%)
Control	100	100	100
ONO3708	100 ± 1.0	101 ± 2.0	101 ± 3.0
S-145	56 ± 1.5	100 ± 1.0	57 ± 2.5

Rat washed platelets (1×10^9 cells/ml) were pretreated with 1 μM ONO3708 or S-145 for 10 min at 37°, and then sedimented at 1200 g for 15 min and suspended in 0.5 ml of resuspension buffer. The platelets were further washed by gel filtration through a column of Sepharose 2B, and resuspended in the resuspension buffer (5×10^8 cells/ml). For the aggregation assay, the pretreated platelets were pre-incubated with 1 mM $CaCl_2$ for 2 min at 37°, and then 40 μg of collagen/ml or 0.4 units/ml thrombin was added. The maximal aggregation reached within 4 min after addition of stimuli was expressed as a percentage of the aggregation response in non-treated platelets. As for the binding assay, the platelets (3.4×10^8) were incubated with 10 nM [^{3}H]U46619 at 24° for 30 min, and the specific binding was determined by displacement with 10 μM U46619. The control value (100%) was defined as the specific binding in non-treated platelets. Data are mean values ± SE for four experiments.

platelet membranes, but not to rat platelet membranes [19]. Why such a difference in sensitivity to the divalent cations occurs is uncertain, but it may reflect a species difference in the characteristics of TXA_2/PGH_2 receptors of human and rat platelets [19].

Scatchard analysis revealed that S-145 bound a single class of recognition sites in human platelet membranes. The maximum number of binding sites for S-145 (2100 fmol/mg protein) was virtually identical with those for SQ29,548 and ONO3708 (Fig. 2), and also comparable to that obtained from U46619 binding studies (1640 fmol/mg) as reported previously [23]. These findings, in addition to the ability of reciprocal displacement by these compounds (Fig. 5), demonstrate that S-145 interacted with the same receptors as those recognized by SQ29,548, ONO3708 and U46619. This conclusion is also supported by high correlations between the potencies of several TXA_2/PGH_2 receptor antagonists to compete with S-145 binding and to suppress the specific bindings for SQ29,548, ONO3708 and U46619 in

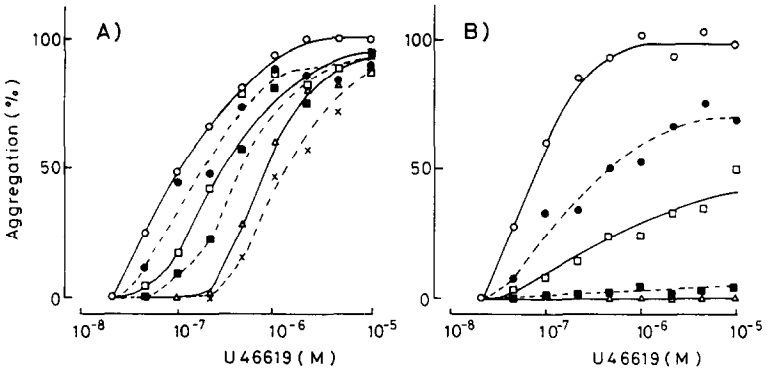

Fig. 9. Concentration-related inhibition by SQ29,548 (A) and S-145 (B) of U46619-induced aggregation of human platelets. Human GFP were preincubated with 1 mM $CaCl_2$ for 2 min at 37° in the absence (○) or the presence of 5 nM (●), 10 nM (□), 20 nM (■), 50 nM (△) or 100 nM (×) of SQ29,548 (A) or S-145 (B). Then various concentrations of U46619 were added. The maximum aggregation reached within 4 min upon addition of U46619 is expressed as a percentage of the aggregation induced by 10 μM U46619 in the absence of these antagonists. Each point is the mean of four experiments.

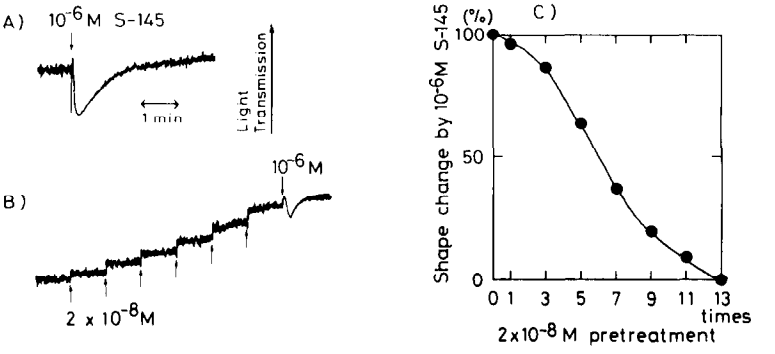

Fig. 10. Abolition of the partial agonistic activity induced by S-145. (A) Human PRP was preincubated for 2 min at 37°, and then 1 μM S-145 was added at the time indicated by the arrow. (B) Human PRP was pretreated with 2×10^{-8} M S-145 six times at 1-min intervals. Next, 1 μM S-145 was added at the time indicated by the arrow. Typical records of at least five experiments are shown. (C) Human PRP was pretreated with 2×10^{-8} M S-145 several times at 1-min intervals, and then 1 μM S-145 was added. The data are expressed as the percentage of the shape change response induced by 1 μM S-145 without pretreatment of 2×10^{-8} M S-145. Each point is the average of the results from four independent experiments.

human platelet membranes (Fig. 5). Further evidence for the specificity of the S-145 binding site is presented by the low affinity of some other eicosanoids, such as PGD_2, PGE_1, $PGF_{2\alpha}$ and TXB_2 (Fig. 3).

Among the three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists, [^{3}H]S-145 had the highest binding affinity for the TXA_2/PGH_2 receptors in human platelets (Fig. 2), which agreed well with the displacement data for the binding of [^{3}H]S-145, [^{3}H]SQ29,548, [^{3}H]ONO3708 and [^{3}H]U46619 (Fig. 5). In fact, S-145 possessed the most potent activity for the blockade of collagen-induced aggregation of rat and human platelets [23]. Stereospecificity for S-145 was observed in competing with the [^{3}H]S-145 binding (Fig. 3) as well as inhibiting the above aggregation responses [23]. Thus, it is clear that the recognition sites of S-145 accurately reflect the biochemical and pharmacological specificities for the platelet TXA_2/PGH_2 receptors.

From the kinetic analysis of receptor bindings in the platelet membranes, the dissociation rate constant (K_{-1}) for S-145 was found to be much smaller than that for other antagonists (Table 1). Thus, such an extremely slow dissociation of S-145 from the receptors may explain several characteristics of the action of S-145 in the platelets.

The extremely smaller K_{-1} values of S-145 in rat, rabbit and human platelets with no large differences in K_1 values gave a smaller K_d compared to SQ29,548 and ONO3708 (Table 1), which may reflect higher affinity of S-145 to platelet TXA_2/PGH_2 receptors with no species differences. However, a wide divergence was observed between the K_d values determined from Scatchard analysis (Fig. 2, 0.75 nM) and kinetic analysis ($K_{-1}/K_1 = 0.16$ nM) for S-145 binding to human platelet membranes, whereas there was a good agreement in SQ29,548 or ONO3708 binding. These discrepancies may be related to some of the partial agonistic characteristics of S-145 [19]

or other mechanisms which remain to be solved.

The observed rate constant (K_{obs}) seems to be in direct proportion to the S-145 concentration, but not to SQ29,548 and ONO3708 (Fig. 7). This is probably because the equation $K_{obs} = K_1[L] + K_{-1}$ can closely resemble $K_{obs} = K_1[L]$ for S-145 binding owing to the extremely small K_{-1} value. The K_{obs} values at the respective K_d values were calculated to be 0.02 min^{-1} for S-145, 0.56 min^{-1} for SQ29,548 and 0.14 min^{-1} for ONO3708. This implies that the association of S-145 to reach 50% occupation of the platelet TXA_2/PGH_2 receptors may be much slower than those of SQ29,548 and ONO3708, suggesting that S-145 requires a longer time to display its full antagonistic activity.

In S-145-pretreated platelets subjected to gel filtration, U46619 binding and collagen-induced aggregation were specifically reduced, whereas no effect was observed in the ONO3708-pretreated platelets (Table 2). These differences represent well the discrepancies in the rate of dissociation from the binding sites between both antagonists (Table 1). Long-lasting action of S-145 was also observed in the antagonism against some TXA_2-mediated responses in *in vivo* studies.* Thus, S-145 can be expected to be a long-lasting TXA_2/PGH_2 receptor antagonist owing to the extremely slow dissociation from the receptors.

The Lineweaver–Burk plot revealed that U46619 and S-145 interacted at the same TXA_2/PGH_2 receptors in a competitive manner (Fig. 4). However, the antagonistic profile of S-145 against the U46619-induced aggregation was apparently non-competitive in contrast to the competitive antagonism of SQ29,548 (Fig. 9). This phenomenon can also be explained by the extremely small dissociation rate constant of S-145. The pretreatment of the platelets with S-145 may cause the long-lasting blockade of the TXA_2/PGH_2 receptors, resulting in the decrease of the receptors available to bind U46619. This characteristic resembles that of acetylcholine in the atropinized preparations [37], in which the antagonism is competitive in the sense that acetylcholine and atropine act on the same site, but the receptors occupied by atropine were unavailable to acetylcholine as if blocked by a non-competitive antagonist owing to the slow rate of dissociation of atropine from its receptors. The abolishment of the partial agonistic action of S-145 by pretreatment of the platelets several times with low concentrations of S-145 or also by a gradual increase of S-145 concentrations (Fig. 10) may be explained in the same manner. Repeated pretreatment of the platelets with low concentrations of S-145 probably causes gradual occupation of the receptors, which may cancel out the partial agonistic action even by addition of a high concentration of S-145. In fact, S-145-induced transient contraction of guinea pig pulmonary smooth muscle could be remarkably diminished or abolished by injecting S-145 at low concentrations or by giving S-145 by the oral route, without the reduction of antagonistic activity.* However, the desensitization of the platelet TXA_2/PGH_2 receptors by S-145 pretreatment should be considered. Recent reports have provided data on the desensitization of TXA_2/PGH_2 receptors against TXA_2/PGH_2 agonist in human platelets caused by an alteration in receptor number and affinity [38, 39]. However, it is difficult for S-145 to examine the desensitization mechanism owing to its non-dissociative characteristic from the receptors.

In conclusion, the binding sites for S-145 on the platelets represent the TXA_2/PGH_2 receptors. The absence of differences in high affinity for the receptors among animal species indicates that S-145 may serve as a new, useful and interesting ligand for furthering our knowledge of the role of the TXA_2/PGH_2 receptors in certain pathophysiological processes. In addition, S-145 can be expected to be a long-lasting TXA_2/PGH_2 receptor-blocking agent for clinical use owing to its extremely slow dissociation from the platelet TXA_2/PGH_2 receptors.

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