Kinetic studies on stereospecific recognition by the thromboxane A₂/prostaglandin H₂ receptor of the antagonist, S-145

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1 The mechanism for the stereospecific recognition of the antagonist S-145 by the thromboxane A₂ (TXA₂)/prostaglandin H₂ (PGH₂) receptor was examined by ligand-binding techniques in rat vascular smooth muscle cells (VSMCs) and in human platelet membranes.

2 Scatchard analysis revealed the existence of a single class of binding sites with the same maximum number for both [³H](+)-S-145 and [³H](−)-S-145 in both cell types. The dissociation constants (*K_d*) for the binding of the (+)-isomer in rat VSMCs and human platelet membranes were, respectively, 0.40 ± 0.03 and 0.20 ± 0.02 nM, each value being lower than that for the (−)-isomer (3.57 ± 0.74 and 2.87 ± 0.08 nM, respectively).

3 The rank orders of potency (*K_i*) for a series of TXA₂/PGH₂ ligands at inhibiting [³H](+)-S-145 binding were highly correlated with those determined for [³H](−)-S-145 binding in both cell preparations.

4 Kinetic analysis of the binding of both radioligands revealed a much lower dissociation rate constant (*k₋₁*) and a slightly greater association rate constant (*k₁*) for the (+)-isomer compared to those for the (−)-isomer.

5 These results suggest that it is at the stage of dissociation from the TXA₂/PGH₂ receptor that the stereochemistry of the optical isomers of S-145 confers their difference in affinity for these receptors in rat VSMCs and human platelet membranes.

Keywords: Thromboxane A₂; thromboxane A₂/prostaglandin H₂ receptor; receptor antagonist; S-145; stereospecificity; rat aortic vascular smooth muscle cells; binding kinetics.

Introduction

Thromboxane A₂ (TXA₂), a metabolite of arachidonic acid, is potent both as an inducer of platelet aggregation and as a vasoconstrictor agent (Hamberg, 1975; Svensson *et al.*, 1977). It is thought to be responsible for the pathogenesis of some cardiovascular and thromboembolic disorders (Halushka & Lefer, 1987; Ogletree, 1987). (±)-S-145, a potent and selective TXA₂/prostaglandin H₂ (PGH₂) receptor antagonist (Narisada *et al.*, 1988), has been successfully used to characterize the TXA₂/PGH₂ receptors in platelets (Hanasaki & Arita, 1988a; Hanasaki *et al.*, 1989a), vascular smooth muscle cells (VSMCs) (Hanasaki *et al.*, 1988a; 1989b) and vascular endothelial cells (Hanasaki *et al.*, 1988b). It has two stereoisomers, and (+)-S-145 has been shown to possess a greater affinity for the TXA₂/PGH₂ receptors than the (−)-isomer (Hanasaki & Arita, 1988b). In addition, (+)-S-145 was observed to be a longer lasting antagonist of some TXA₂-mediated pharmacological responses both *in vivo* (Hori *et al.*, 1989) and *in vitro* (Nakajima & Ueda, 1989; Hanasaki *et al.*, 1989b). Similar stereospecific recognition of optical isomers has been reported for various types of receptors (Burgisser *et al.*, 1981); however, little is known about the underlying mechanism.

In the present study, we examined the differences in the binding mode between [³H](+)-S-145 and [³H](−)-S-145 in rat VSMCs and human platelet membranes. This is the first report of a kinetic analysis of the stereospecific recognition by TXA₂/PGH₂ receptors of the optical isomers of S-145.

Methods

Chemical preparation of [³H]-S-145

(−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-[4-³H]-phenyl sulphonyl-amino-bicyclo[2.2.1]hept-2-yl)hept-5-enoic acid sodium salt,

[³H](−)-S-145 sodium salt, was prepared as follows. To a stirred solution of 40 mg (0.18 mmol) methyl (−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-aminobicyclo[2.2.1]hept-2-yl)hept-5-enoate (Narisada *et al.*, 1988) in anhydrous benzene (2 ml) were added 20 mg (0.2 mmol) triethylamine and a solution of [4-³H]-phenylsulphonyl chloride (100 mCi, 23.7 Ci mmol^{−1}, Amersham) in anhydrous benzene (11.5 ml). The mixture was concentrated *in vacuo* (60 mmHg) at 35°C to about 2 ml and stirred for 2.5 h at room temperature. The reaction mixture was evaporated *in vacuo* below 25°C, leaving a viscous oil which was purified by column chromatography on silica gel (Merck No. 7734, 900 mg; elution with benzene-ethylacetate 9:1), giving pure methyl (−)-(1S, 2R, 3R, 4R)-(5Z)-7-(3-[4-³H]-phenylsulphonylaminobicyclo[2.2.1]hept-2-yl)hept-5-enoate ([³H](−)-S-145 methyl ester) (80 mCi, 0.0033 mmol) as a viscous oil. [³H](−)-S-145 methyl ester was converted into [³H](−)-S-145 sodium salt by saponification with 1 N sodium hydroxide (0.1 ml) in methanol (0.8 ml) by means of stirring for 15 h at room temperature. The mixture was then evaporated *in vacuo* below 25°C, giving crude [³H](−)-S-145 sodium salt as a solid residue which was purified by chromatography (Waters SEP-PAK_{C-18}; elution with ethanol-water 1:4). The fractions containing the compound required in almost pure form were combined and evaporated *in vacuo* to dryness, giving [³H](−)-S-145 sodium salt (71.5 mCi, 1.2 mg, 24.07 Ci mmol^{−1}, radiochemical purity 99.9%) in a 71.5% yield based on [4-³H]-phenylsulphonyl chloride. The sodium salt of [³H](−)-S-145 was dissolved in 95% ethanol, and the radioactive concentration was adjusted to 1.0 mCi ml^{−1}. Radiochemical purity was measured by thin layer chromatography (t.l.c.) autoradiography followed by liquid scintillation counting and high performance liquid chromatography (h.p.l.c.) (Nucleosil 5C₁₈, 4.6 mm × 15 cm; mobile phase, CH₃CN:CH₃OH:H₂O:CH₃COOH = 300:200:300:1; detection, u.v. 220 nm and radioactivity measured with a Packard Trace II-7150 detector). The intermediate and product were identified with authentic unlabelled compounds by comparison of t.l.c. (*R_F*) and h.p.l.c. (retention time). The *R_F* value of

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(-)-S-145 was 0.31 with chloroform/methanol (95/5, v/v) as the solvent system, and the retention time obtained by h.p.l.c. analysis was 7.3 min at the flow rate of 1.5 ml min⁻¹.

(+)-(1R, 2S, 3S, 4S)-(5Z)-7-(3-[4-³H]-phenylsulphonylaminobicyclo[2.2.1]hept-2-yl)hept-5-enoic acid sodium salt, [³H]-(+)-S-145 sodium salt (31.9 mCi, 0.48 mg, 26.4 Ci mmol⁻¹, radiochemical purity 99.9%) was prepared from 27 mg (0.114 mmol) methyl (+)-(1R, 2S, 3S, 4S)-(5Z)-7-(3-aminobicyclo[2.2.1]hept-2-yl)hept-5-enoate (Narisada *et al.*, 1988) and [4-³H]-phenylsulphonylchloride (99 mCi, 29 Ci mmol⁻¹, Amersham) by an essentially similar method to that used for the synthesis of [³H]-(-)-S-145 sodium salt.

Preparation of cultured vascular smooth muscle cells of rat aorta and human platelet membranes

Cultured rat aortic VSMCs were prepared as described previously (Hanasaki *et al.*, 1988a). Briefly, the cells were isolated from the medial explants of the aorta of 7-week-old male Sprague-Dawley rats by the method of Ross (1971). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal calf serum (Gibco, Grand Island, NY), penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The preparation of human platelet membranes was carried out as described by Hanasaki *et al.* (1989a). In brief, human blood, drawn by venepuncture from healthy male volunteers, was placed in 0.15 vol. of acid citrate dextrose (85 mM trisodium citrate, 70 mM citric acid and 110 mM glucose) containing 12 µg ml⁻¹ PGE₁. Platelet-rich plasma (PRP) was obtained by centrifugation at 160 *g* for 10 min and the platelets were then 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). After homogenization followed by centrifugation at 30,000 *g* for 10 min at 4°C, the pellets were suspended in incubation buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 10 µM indomethacin and 0.3 mM PMSF) to give a protein concentration of 2 mg ml⁻¹ and stored at -80°C until use. Protein concentration was determined by the method of Lowry *et al.* (1951) with human serum albumin as a standard.

Binding assays

For the binding assays with rat VSMCs, confluent VSMCs in 35 mm diameter dishes (approximately 5 × 10⁵ cells/dish) were washed twice with phosphate-buffered saline. The incubations were carried out at 25°C in culture dishes in a total volume of 1 ml binding medium (Hanks' solution, pH 7.6, containing 0.1% BSA) with each tritium-labelled TXA₂/PGH₂ receptor ligand at the indicated concentration. After the reaction was stopped by rapid removal of the medium, the cells were washed three times with ice-cold 0.9% NaCl, and then solubilized in 1 N NaOH. The content of radioactivity was analyzed after adding an equal volume of 1 N HCl. The specific binding is defined as the difference between binding in the presence and absence of the unlabelled antagonist (10 µM). The specific binding of 2.0 nM [³H]-(+)-S-145 and 10.5 nM [³H]-(-)-S-145, respectively, represented 90 and 70% of the total binding. The binding assays in human platelet membranes were carried out according to a method described previously (Hanasaki *et al.*, 1989a). Briefly, platelet membranes (80 µg) were incubated with each tritium-labelled TXA₂/PGH₂ receptor ligand in a total volume of 0.5 ml at 25°C. After the incubation, ice-cold 0.9% NaCl 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 three times with ice-cold 0.9% NaCl, and analyzed for radioactivity. The specific binding of 3.6 nM [³H]-(+)-S-145 and 11.2 nM [³H]-(-)-S-145, respectively, represented 96 and 92% of the total binding.

Materials

U46619, PGE₁, PGE₂, PGD₂, PGF_{2α} and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TXB₂ was purchased from Funakoshi (Osaka, Japan). Phenylmethylsulphonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) were from Nakarai Chemicals (Kyoto, Japan). (±)-S-145, and its (+)- and (-)-isomers, were synthesized at Shionogi Research Laboratories, Osaka (Narisada *et al.*, 1988). 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-pentanol-15-epi-TXA₂) was prepared as described previously (Hanasaki *et al.*, 1989a). Other chemicals were of analytical grade and were obtained from commercial sources.

Statistical analysis

Linear regression analysis of the binding data was performed according to standard methods (Nelder & Mead, 1965).

Results

Equilibrium binding studies of [³H]-(+)-S-145 and [³H]-(-)-S-145

The characteristics of the equilibrium binding of [³H]-(+)-S-145 and [³H]-(-)-S-145 were first examined. Saturation

Figure 1 Binding of [³H]-S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes. Saturation curves for the equilibrium binding of [³H]-(+)-S-145 (○) or [³H]-(-)-S-145 (●) to rat VSMCs (a) and human platelet membranes (c) are shown. Rat VSMCs were incubated with increasing concentrations of [³H]-(+)-S-145 or [³H]-(-)-S-145 at 25°C for 90 and 60 min, respectively. Human platelet membranes were incubated with increasing concentrations of [³H]-(+)-S-145 or [³H]-(-)-S-145 at 25°C for 40 and 30 min, respectively. Non-specific binding in the presence of 10 µM unlabelled ligand was subtracted from each point, which is representative of duplicate determinations in three experiments; (b) and (d) are the Scatchard plots of the same data as in (a) and (c), respectively, showing the lines obtained by linear regression.

Table 1 Comparison of K_d and B_{max} values for binding of the isomers of S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes

Ligand	K_d (nM)	B_{max} (fmol)
<i>Rat VSMCs</i>		
[3H](+)-S-145	0.40 ± 0.03	48.7 ± 8.9 per 10^6 cells
[3H](−)-S-145	3.57 ± 0.74	44.8 ± 1.7
<i>Human platelet membranes</i>		
[3H](+)-S-145	0.20 ± 0.02	1970 ± 36 mg $^{-1}$ protein
[3H](−)-S-145	2.87 ± 0.08	1880 ± 82

Values were obtained by Scatchard analysis. Data are means $\pm$ s.e.mean of 3 experiments.

studies of the binding to rat VSMCs and human platelet membranes revealed that non-specific binding increased linearly with rising concentrations of each ligand, whereas the specific binding was saturable in both cell preparations (Figure 1a,c). Scatchard analysis of these data indicated the existence of a single class of binding sites for each optical isomer of S-145 in rat VSMCs as well as human platelet membranes (Figure 1b,d). The K_d value for the [3H](+)-isomer was almost 10 times smaller than that of the [3H](−)-isomer (Table 1). The maximum number of binding sites (B_{max}) was

Table 2 Inhibition of [3H](+)-S-145 and [3H](−)-S-145 binding to rat vascular smooth muscle cells (VSMCs) and human platelet membranes by thromboxane A_2 (TXA $_2$)/prostaglandin H_2 (PGH $_2$) receptor ligands

Ligand	K_i (nM)	
	[3H](+)-S-145	[3H](−)-S-145
<i>Rat VSMCs</i>		
(+)-S-145	0.23 ± 0.08	0.32 ± 0.06
(±)-S-145	0.29 ± 0.07	0.41 ± 0.02
(−)-S-145	1.7 ± 0.3	1.7 ± 0.1
ONO3708	7.3 ± 0.3	7.9 ± 1.5
U46619	11.3 ± 1.6	20.7 ± 5.0
<i>Human platelet membranes</i>		
(+)-S-145	0.25 ± 0.02	0.20 ± 0.06
(±)-S-145	0.45 ± 0.03	0.41 ± 0.14
(−)-S-145	4.7 ± 0.2	3.2 ± 1.0
ONO3708	5.0 ± 0.3	3.9 ± 1.2
U46619	52.7 ± 1.4	49.8 ± 9.7

The K_i values were determined according to the equations of Cheng & Prusoff (1973), using the IC_{50} values derived from the inhibition data (Figure 2) and the K_d values obtained by Scatchard analysis (Figure 1). Data are means $\pm$ s.e.mean of 3 experiments. The correlation coefficients between the negative logarithms of the K_i values of the five TXA $_2$ /PGH $_2$ receptor ligands against [3H](+)-S-145 binding and against [3H](−)-S-145 binding were 0.99 in both rat VSMCs and human platelet membranes.

found to be almost the same for the optical isomers of S-145 in each preparation.

Binding specificity of [3H](+)-S-145 and [3H](−)-S-145 in rat vascular smooth muscle cells and human platelet membranes

Several structurally dissimilar compounds were examined for their abilities to compete with the specific binding of [3H](+)-S-145 or [3H](−)-S-145. Five TXA $_2$ /PGH $_2$ receptor ligands inhibited the binding of both [3H](+)-S-145 (Figure 2a) and [3H](−)-S-145 (data not shown) to rat VSMCs in a concentration-dependent manner, and the potency series, as given by the IC_{50} values for unlabelled S-145, was found to be (+)-isomer > racemate > (−)-isomer. U46619, a stable TXA $_2$ -mimetic agonist (Coleman *et al.*, 1980), also completely inhibited binding of both ligands, whereas PGD $_2$, PGE $_2$, PGF $_{2\alpha}$ and TXB $_2$ were inhibitory only at concentrations greater than $10 \mu M$, demonstrating the binding selectivity of both optical isomers for TXA $_2$ /PGH $_2$ receptors. Similar displacement data were obtained with human platelet membranes (Figure 2b). The IC_{50} values for the respective ligands in both cell preparations were obtained from the displacement data, and then the K_i values, given in Table 2, were calculated by the Cheng-Prusoff equation (Cheng & Prusoff, 1973), using the respective K_d values shown in Table 1. The negative logarithms of the K_i values of the five TXA $_2$ /PGH $_2$ receptor ligands against [3H](+)-S-145 binding were highly correlated with the corresponding K_i values for inhibition of [3H](−)-S-145 binding in both rat VSMCs ($r = 0.99$) and human platelet membranes ($r = 0.99$).

Kinetic analysis of the binding of [3H](+)-S-145 and [3H](−)-S-145 to rat vascular smooth muscle cells and human platelet membranes

In order to characterize further the binding of [3H](+)-S-145 and [3H](−)-S-145, kinetic analysis was performed with rat VSMCs and human platelet membranes. As shown in Figure 3a, the specific binding of 1.9 nM [3H](+)-isomer and 10.4 nM [3H](−)-isomer to rat VSMCs followed similar time-

Figure 2 Inhibition of specific [3H](+)-S-145 binding to rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (b) by S-145 and related compounds. Rat VSMCs were incubated with 2.0 nM [3H](+)-S-145 in the presence of various concentrations of (+)-S-145 ($\circ$), ($\pm$)-S-145 ($\bullet$), (−)-S-145 ($\square$), ONO3708 ($\blacksquare$), U46619 ($\triangle$), prostaglandin D_2 (PGD $_2$) ($\blacktriangle$), PGF $_{2\alpha}$ (∇), PGE $_2$ ($\blacktriangledown$) and TXB $_2$ ($\diamond$). Incubation was carried out at $25^\circ C$ for 90 min. Human platelet membranes were incubated with 1.8 nM [3H](+)-S-145 at $25^\circ C$ for 40 min in the presence of the same compounds. The control value (100%) was defined as the specific binding of [3H](+)-S-145 in the absence of these compounds. Each point is mean of three independent experiments performed in triplicate; vertical bars show s.e.mean.

Figure 3 The time course of association of [^3H]-S-145 to rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (c). Rat VSMCs were incubated with 1.9 nM [^3H]-(+)-S-145 (○) or 10.4 nM [^3H]-(-)-S-145 (●) at 25°C for the indicated times. Human platelet membranes were incubated with 3.5 nM [^3H]-(+)-S-145 or 11.0 nM [^3H]-(-)-S-145 at 25°C. The specific binding is expressed as a percentage of the specific binding at equilibrium. B_{eq} is the specific binding at equilibrium and B_t is the amount of [^3H]-S-145 specifically bound at any indicated time. Each point is the mean of three independent experiments; vertical bars show s.e.mean; (b) and (d) are the pseudo first-order rate plots of the data taken from (a) and (c), respectively; the slope of this plot is k_{obs} .

dependent processes and reached equilibrium at 65 and 50 min, respectively. The observed rate constants (k_{obs}), derived from the slope of the pseudo first-order rate plot of these data (Figure 3b), were found to be $0.040 \pm 0.006 \text{ min}^{-1}$ for the [^3H]-(+)-isomer and $0.061 \pm 0.005 \text{ min}^{-1}$ for the

[^3H]-(-)-isomer. Similar association experiments were performed with human platelet membranes and the k_{obs} values were calculated to be $0.24 \pm 0.06 \text{ min}^{-1}$ for 3.5 nM [^3H]-(+)-S-145 and $0.47 \pm 0.01 \text{ min}^{-1}$ for 11.0 nM [^3H]-(-)-S-145 (Figure 3c,d). Next, to determine the dissociation rate constant

Figure 4 The time course of dissociation of [^3H]-S-145 from rat vascular smooth muscle cells (VSMCs) (a) and human platelet membranes (c). The association between rat VSMCs and 1.9 nM [^3H]-(+)-S-145 (○) or 10.4 nM [^3H]-(-)-S-145 (●) was carried out at 25°C for 90 and 60 min, respectively, and that of human platelet membranes with 3.5 nM [^3H]-(+)-S-145 or 11.0 nM [^3H]-(-)-S-145 was carried out at 25°C for 40 and 30 min, respectively. Next, 10 μM unlabelled ligand was added at time zero to initiate displacement of the ^3H -labelled antagonist from its specific binding site. The data are expressed with the value of the specific binding at time zero as 100%. B_t is the amount of [^3H]-S-145 specifically bound at any particular time, t , and B_0 is the amount at time zero. Each point is the mean of three independent experiments; vertical bars show s.e.mean; (b) and (d) are the linear transformation of the data taken from (a) and (c), respectively; the slope of the plot is k_{-1} .

Table 3 Kinetic, and derived equilibrium, constants for the binding of ^3H -labelled (+)-S-145 and (-)-S-145 to rat vascular smooth muscle cells (VSMCs) and human platelet membranes

Ligand	k_1 ($\text{M}^{-1} \text{min}^{-1}$)	k_{-1} (min^{-1})	$K_d = k_{-1}/k_1$ (nM)
<i>Rat VSMCs</i>			
$[^3\text{H}](+)\text{-S-145}$	$2.4 \pm 0.4 \times 10^7$	0.0010 ± 0.0003	0.039 ± 0.005
$[^3\text{H}](-)\text{-S-145}$	$4.8 \pm 0.3 \times 10^6$	0.015 ± 0.003	3.1 ± 0.5
<i>Human platelet membranes</i>			
$[^3\text{H}](+)\text{-S-145}$	$6.7 \pm 0.9 \times 10^7$	0.0028 ± 0.0003	0.043 ± 0.004
$[^3\text{H}](-)\text{-S-145}$	$3.0 \pm 0.2 \times 10^7$	0.14 ± 0.01	4.5 ± 0.3

The results shown are means $\pm$ s.e.mean of 3 experiments obtained in triplicate determinations. Experiments were performed as described in the legends of Figure 3 and Figure 4.

(k_{-1}), both cell preparations were incubated with each ^3H -labelled antagonist to ensure that the equilibrium had been reached. At an arbitrary zero time point, an excess of the respective unlabelled isomer of S-145 ($10 \mu\text{M}$) was added, and the time course of displacement of each radioligand from its binding site was determined. As shown in Figure 4a, in rat VSMCs the dissociation of $[^3\text{H}](+)\text{-S-145}$ from its specific binding sites was much slower than that of $[^3\text{H}](-)\text{-S-145}$. The times at which one-half of its equilibrium level had been reached were 960 min for the (+)-isomer and 50 min for the (-)-isomer, and the times at which complete dissociation had been achieved were, respectively, 72 and 2.5 h (data not shown). Similar dissociation patterns of $[^3\text{H}](+)\text{-S-145}$ and $[^3\text{H}](-)\text{-S-145}$ were observed with human platelet membranes (Figure 4c). Linear transformation of these data (Figure 4b,d) gave a k_{-1} value for the binding of each radioligand, and we calculated the value for k_1 (association rate constant), according to the equation $k_1 = (k_{\text{obs}} - k_{-1})/[L]$ (where $[L]$ is radioligand concentration), and also determined the K_d value given by $K_d = k_{-1}/k_1$, all of which are summarized in Table 3. An extremely slow k_{-1} as well as a slightly greater k_1 value for the (+)-isomer were observed for both cell preparations.

Discussion

The present study describes a kinetic analysis for the stereospecific recognition by $\text{TXA}_2/\text{PGH}_2$ receptors of the optical isomers of S-145. We used cultured VSMCs from rat thoracic aorta and human platelet membranes as *in vitro* models since these cell preparations possess intrinsic $\text{TXA}_2/\text{PGH}_2$ receptors which, when activated, lead to the corresponding pharmacological responses (Ogletree, 1987; Hanasaki *et al.*, 1989b). However, in this study, the binding experiments for rat VSMCs were performed with cells which were adherent to culture dishes, instead of the trypsin-harvested cells which had been used previously (Hanasaki *et al.*, 1989b), since the treatment with trypsin and chymotrypsin causes destruction of the $\text{TXA}_2/\text{PGH}_2$ receptor-mediated responses in platelets (Lukasiewicz *et al.*, 1989). Scatchard analysis proved that the receptor density determined by the present method was about 5 fold greater than the levels obtained by the previous methods (Hanasaki *et al.*, 1989b) and showed that this occurred without any significant changes in the ligand affinities, thus demonstrating similar tryptic proteolysis of the $\text{TXA}_2/\text{PGH}_2$ receptors in rat VSMCs.

Scatchard analysis, as well as the displacement data, clearly showed that the binding affinity of $[^3\text{H}](+)\text{-S-145}$ for $\text{TXA}_2/\text{PGH}_2$ receptors was much greater than that of the (-)-isomer, which is consistent with the pharmacological data in

vascular smooth muscle as well as in platelets (Hanasaki *et al.*, 1989b). Similar radioligand binding studies on the differences in the binding affinity between stereoisomers have been performed with several receptor systems (Burgisser *et al.*, 1981). For the γ -aminobutyric acid (GABA) receptor, Drew *et al.* (1984) reported different classes of binding sites for $[^3\text{H}](-)$ - and $[^3\text{H}](+)\text{-baclofen}$, a GABA agonist, in synaptic membranes of rat cerebellum, which might account for their discrepancies in pharmacological potencies. However, for the optical isomers of S-145, Scatchard analysis revealed that the maximum number of binding sites for the (+)-isomer was virtually identical to that for the (-)-isomer (Table 1), suggesting that both isomers interact with the same binding sites. This conclusion is further supported by the high correlation between the inhibitory potencies (K_i) of several $\text{TXA}_2/\text{PGH}_2$ receptor ligands when competing with the binding of $[^3\text{H}](+)\text{-}$ and $[^3\text{H}](-)\text{-S-145}$ (Table 2). Thus, the disparities between the stereoisomers of S-145 appear to arise from differences in their binding modes to the same $\text{TXA}_2/\text{PGH}_2$ receptor molecule.

The kinetic analysis of the radioligand binding in both cell preparations (Figures 3 and 4) revealed that the (+)-isomer possesses a slightly greater k_1 value than the (-)-isomer, which was almost the same as those of other $\text{TXA}_2/\text{PGH}_2$ antagonists, such as ONO3708 and SQ29,548 (Hanasaki *et al.*, 1989a). On the other hand, the dissociation rate constant (k_{-1}) for the (+)-isomer was found to be much smaller than that for the (-)-isomer as well as for other receptor antagonists (Hanasaki *et al.*, 1989a), which is consistent with the long-lasting action of (+)-S-145 in the antagonism of some TXA_2 -mediated pharmacological responses *in vivo* (Hori *et al.*, 1989). These findings suggest that these $\text{TXA}_2/\text{PGH}_2$ receptors would strictly recognize the differences between the optical isomers of S-145 at the stage of dissociation from the receptors rather than at the stage of accessibility to the receptors.

The very much smaller k_{-1} value for the (+)-isomer would make a large contribution towards it having a much lower K_d value ($K_d = k_{-1}/k_1$) than that for the (-)-isomer, which would reflect the higher affinity of the (+)-isomer for the $\text{TXA}_2/\text{PGH}_2$ receptors in both preparations. However, a wide divergence (approximately 10 fold) was observed between the K_d values derived from kinetic analysis and those from Scatchard analysis for (+)-S-145 binding in each preparation, whereas a good correlation was observed for (-)-S-145 binding. Such a great difference between both the K_d values was also reported in the binding of ($\pm$)-S-145 which acts as a partial agonist to evoke shape change without aggregation and secretion responses in human platelets (Hanasaki *et al.*, 1989a). Our preliminary experiments have shown that (+)-S-145 also possesses higher partial agonist potency (by about ten times) than (-)-S-145 in human platelets (unpublished data). These observations might suggest the possibility that the discrepancy between the K_d values determined from kinetic analysis and Scatchard analysis arise from some conformational change in the $\text{TXA}_2/\text{PGH}_2$ receptors after the (+)-S-145 has bound which results from its potent partial agonist activity. Recently, conformational analyses verified that ($\pm$)-S-145 has energetically stable conformers similar to the putative active form (hairpin-like structure) of TXA_2 or U46619 (Ezumi *et al.*, 1990). Such a stereochemical comparison would offer further information about the mechanism for the differences in the dissociation pattern of the optical isomers of S-145. Since the $\text{TXA}_2/\text{PGH}_2$ receptor has been purified from human platelets (Ushikubi *et al.*, 1989) and its amino acid sequence has recently been determined (Hirata *et al.*, 1991), studies on the structure of its binding domains should also help further our knowledge of the interaction of (+)-S-145 with the $\text{TXA}_2/\text{PGH}_2$ receptor at the molecular level.

In conclusion, this paper presents the characterization of the stereospecific recognition of the $\text{TXA}_2/\text{PGH}_2$ receptor for the optical isomers of S-145 by kinetic analysis. The absence

of differences in high affinity binding of (+)-S-145 for the receptors between platelets and VSMCs indicated that (+)-S-145 may serve as a useful ligand for analysis of the role of TXA₂/PGH₂ receptors in certain pathophysiological processes. In addition, (+)-S-145 can be expected to be a long-lasting TXA₂/PGH₂ receptor antagonist for clinical use owing

to its extremely slow dissociation from the TXA₂/PGH₂ receptors.

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