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BIOCHEMICAL CHARACTERIZATION AND COMPARISON OF RAT THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS IN PLATELETS AND CULTURED AORTIC SMOOTH MUSCLE CELLS

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Abstract—Comparison of thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors in rat cultured vascular smooth muscle cells (VSMC) with those in rat gel-filtered platelets (GFP) was carried out using a receptor-ligand binding assay. The binding of each of three radiolabeled TXA_2/PGH_2 receptor antagonists ([^{3}H]S-145,[^{3}H]SQ29,548, and [^{3}H]ONO3708) displayed high affinity and specificity as well as saturable and displaceable binding to a single class of recognition sites with the same maximum number in both VSMC and GFP. The K_d values for [^{3}H]S-145 were almost identical for both VSMC and GFP, whereas the values for [^{3}H]SQ29,548 and [^{3}H]ONO3708 for VSMC were approximately two and six times larger than that for GFP. Kinetic analysis of the binding of each receptor antagonist revealed a smaller K_1 value (the association rate constant) for [^{3}H]SQ29,548 and a larger K_{-1} value (the dissociation rate constant) for [^{3}H]ONO3708 for VSMC compared to GFP, in contrast with almost the same kinetic constants for the [^{3}H]S-145 binding for both cells. Comparison of the inhibitory potencies (K_i values) for [^{3}H]S-145 binding for both VSMC and GFP proved that S-145 had the same affinity for both cells; ONO11120 and BM13177, as well as SQ29,548 and ONO3708, possessed lower affinity for VSMC; and U46619 exhibited higher affinity for VSMC. The rank orders of potency were identical in both cells (S-145 > SQ29,548 > ONO3708 > ONO11120 > BM13177), which correlated well with their pharmacological activities. These results suggest a similarity in ligand binding specificity with some differences in the accessibility of the antagonists in the TXA_2/PGH_2 receptors between platelets and vascular smooth muscles.

Thromboxane A_2 (TXA_2),† a product of arachidonic acid metabolism in blood platelets, is among the most potent naturally occurring constrictors of vascular smooth muscles and inducers of platelet aggregation [1, 2], and thus is thought to play a central role in the mediation of a number of hypoxic and ischemic states [3, 4]. A variety of stable analogues, which selectively inhibit or mimic its biological activities, have been synthesized as tools for investigating the mechanisms of TXA_2 action and the nature of putative TXA_2/PGH_2 receptors in both platelets and vascular preparations [5, 6]. Pharmacological studies using a series of receptor antagonists and agonists raised the possibility of the existence of distinct classes of TXA_2/PGH_2 receptors between platelets and blood vessels [7–11], but their biochemical characteristics could not be defined by ligand binding studies. Platelet TXA_2/PGH_2 receptors have been well characterized by binding studies using several radiolabeled ligands, such as [^{3}H]U46619 [12, 13], [^{3}H]13-azaprostanoic acid [14], [^{125}I]PTA-OH [15–18], [^{125}I]-*p*-OH-SQ28,668 [19] and [^{3}H]SQ29,548 [20], on intact human platelets and also on the solubilized form from platelet membranes [21, 22]. More recently, we succeeded in identifying the specific TXA_2/PGH_2 receptors on cultured rat aortic smooth muscle cells (VSMC) as well as on rat platelets by means of binding studies [23, 24]. This cultured VSMC may provide a direct means for distinguishing between TXA_2/PGH_2 receptors on platelets and smooth muscles of the same species.

In this study, we used some specific radiolabeled TXA_2/PGH_2 receptor antagonists with widely dissimilar structures, such as SQ29,548 [25], ONO3708 [26] and S-145 [27]. S-145 is a newly synthesized receptor antagonist with a partial agonistic activity for both platelets and vascular smooth muscle preparations [28, 29]. First, we tried to find differences between the rat platelet and VSMC TXA_2/PGH_2 receptors by comparing kinetic parameters describing the binding of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708. Second, we compared inhibitory potencies against [^{3}H]S-145 binding in both types of cells using several types of TXA_2/PGH_2 receptor antagonists.

This paper provides the first biochemical characterization of the TXA_2/PGH_2 receptors on platelets and VSMC by definite classification of receptor antagonists with different binding affinities to both types of cells.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]SQ29,548 (40.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Collagen (type IV, soluble), U46619, PGE_1, PGD_2, $PGF_{2\alpha}$ and bov-

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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; VSMC, vascular smooth muscle cells; and Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

ine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO). TXB_2 was purchased from Funakoshi (Osaka, Japan). S-145 [(±)-5(Z)-7-[3-*endo*-phenylsulfonylamino [2.2.1]bicyclohept-2-*exo*-yl] - heptenoic acid] [29], 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) [30], ONO3708 [(9,11),(11,12) - dideoxa - 9α,11α-dimethylmethano-11,12 - methano - 13,14 - dihydro-12-aza-14-oxo-15-cyclo - pentyl-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-15$\alpha\beta$-ω-tetranor-TXA_2) [31] and BM13177 (4-[2-(benzenesulfonamido)-ethyl]phenoxyacetic acid) [32] were synthesized in the Shionogi Research Laboratories (Osaka, Japan). 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; this concentration of DMSO had no effect on the binding experiments. 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]benzenesulfonyl 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 (±)-5(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°. Then the mixture was 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 almost pure [^{3}H]S-145 were combined and evaporated *in vacuo* to dryness, giving [^{3}H]S-145 sodium salt (41.7 mCi, 0.64 mg, 24.5 Ci/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 being 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–éthyl 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 previously 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 514 μCi/ml ethanol solution.

Preparation of rat gel-filtered platelets (GFP)

Blood was collected from Sprague–Dawley rats 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% BSA [33]. 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% BSA, pH 7.35). Platelets were separated from plasma proteins by gel filtration through a column of Sepharose 2B, and suspended in the resuspension buffer to a final concentration of 5×10^8 cells/ml.

Culture of smooth muscle cells (VSMC) of rat aorta

Aortic smooth muscle cells were isolated from the medial explants of the thoracic aorta of 7-week-old

male Sprague–Dawley rats by the method of Ross [34]. The cells were maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2, 95% O_2 at 37°. Cells were harvested with 0.125% trypsin–0.01% EDTA and subcultured at a 1:4 dilution when their growth reached confluency. Cells displayed multilayered growth and abundant myofilament characteristics of smooth muscle cells [34]. After serial subculture, VSMC harvested between the 5th and 8th passage were used in the experiments.

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

Contraction of rat thoracic aorta

Sprague–Dawley rats weighing 220–250 g were killed, and a 4-cm segment of the thoracic aorta was excited. After removal of adhering fat and connective tissue, helical strips were prepared according to the method of Furchgott and Bhadrakom [35]. Each strip (20 × 3 mm) was mounted in a 12-ml water-jacketed muscle chamber maintained at 37° and bubbled with 95% O_2–5% CO_2 in Krebs–Henseleit solution containing 1 μM indomethacin. After equilibrium for 1 hr at 1 g of initial tension, the isometric tension development was measured after adding KCl (30 mM) as a reference compound, which was recorded on a Grass polygraph (model 7) via force-displacement transducers (FT-03). The ED_{50} value for U46619 to provoke contraction was 7.8 nM, and the nearly maximum contraction was induced with 30 nM U46619. To examine the antagonism of U46619-induced contraction, various concentrations of antagonists were preincubated for 30 min, and then 30 nM U46619 was added and the contractile response was measured. The IC_{50} values for the respective antagonists were calculated from the normalized concentration–response curves by linear regression analysis.

Binding experiments

As for GFP, incubations were carried out in a 0.4-ml final volume which contained GFP (1.7×10^8 platelets) and each [^{3}H]-labeled TXA_2/PGH_2 receptor antagonist dissolved in the resuspension buffer. Specific binding is defined as the difference between binding in the presence and absence, respectively, of a 10 μM concentration of the unlabeled antagonist. The specific binding of [^{3}H]S-145 (2.2 nM), [^{3}H]SQ29,548 (2.5 nM) and [^{3}H]ONO3708 (5.2 nM) represented, respectively, 92 ± 3, 98 ± 1 and 84 ± 3% of the total binding to rat GFP. For the binding study with VSMC, confluent VSMC were washed twice with phosphate-buffered saline and then harvested in 0.125% trypsin solution containing 0.01% EDTA. After washing twice with DMEM, VSMC were suspended in Hanks' medium, pH 7.6, containing 0.1% BSA (binding medium). Binding study was performed by incubating VSMC (2×10^6 cells) with the above ^{3}H-labeled antagonists in 0.5 ml of binding medium at 24°. Specific binding of [^{3}H]S-145 (5.8 nM), [^{3}H]SQ29,548 (4.7 nM) and [^{3}H]ONO3708 (23.5 nM) represented, respectively, 83 ± 2, 83 ± 3 and 32 ± 4% of the total binding to rat VSMC. For the competition studies, GFP or VSMC were incubated with [^{3}H]S-145 in the presence of increasing concentrations of various compounds at 24° for 120 min. 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. Radioactivity was measured using a scintillation counter (Aloka, LSC-700).

Statistical analysis

Linear regression of the binding data was performed according to the standard methods [36].

RESULTS

Binding of [^{3}H]S-145, [^{3}H]SQ29,548 or [^{3}H]ONO3708 to rat cultured vascular smooth muscle cells (VSMC) and rat gel-filtered platelets (GFP)

In preliminary experiments, total, specific, or non-specific binding of [^{3}H]S-145 (5.8 nM) was found to increase linearly over VSMC ranging from 0.5×10^6 to 3.0×10^6 cells. There was good linearity between the extent of the specific binding of [^{3}H]S-145 (2.5 nM) and the platelet content over a wide range (0.4×10^8 to 3.4×10^8 cells) of rat GFP. All subsequent binding assays were carried out with VSMC or GFP of 2.0×10^6 and 1.7×10^8 cells respectively.

Kinetic characteristics of [^{3}H]S-145, [^{3}H]SQ29,548 or [^{3}H]ONO3708 binding to VSMC and GFP

As shown in Fig. 1, specific binding of each ^{3}H-labeled TXA_2/PGH_2 receptor antagonist to cultured VSMC or GFP at 24° was a time-dependent process. In VSMC (Fig. 1A), the association of [^{3}H]SQ29,548 (4.7 nM) or [^{3}H]ONO3708 (23.5 nM) was rapid, reaching equilibrium levels within 10 min and remaining unchanged for the subsequent 120 min, whereas that of [^{3}H]S-145 (5.8 nM) was much slower and the equilibrium binding was reached at 45 min and remained at the same level for up to 210 min. The association of [^{3}H]S-145 (2.5 nM) to GFP was also slower than that of [^{3}H]SQ29,548 (2.5 nM) or [^{3}H]ONO3708 (5.2 nM) (Fig. 1B), and remained at the equilibrium level for the subsequent 160 min. These results indicate that neither a loss of binding sites nor degradation of radioligand occurred within the incubation time. The observed rate constant (K_{obs}), derived from the slope of the pseudo first-order rate plot of these data (Fig. 1, inset), was found

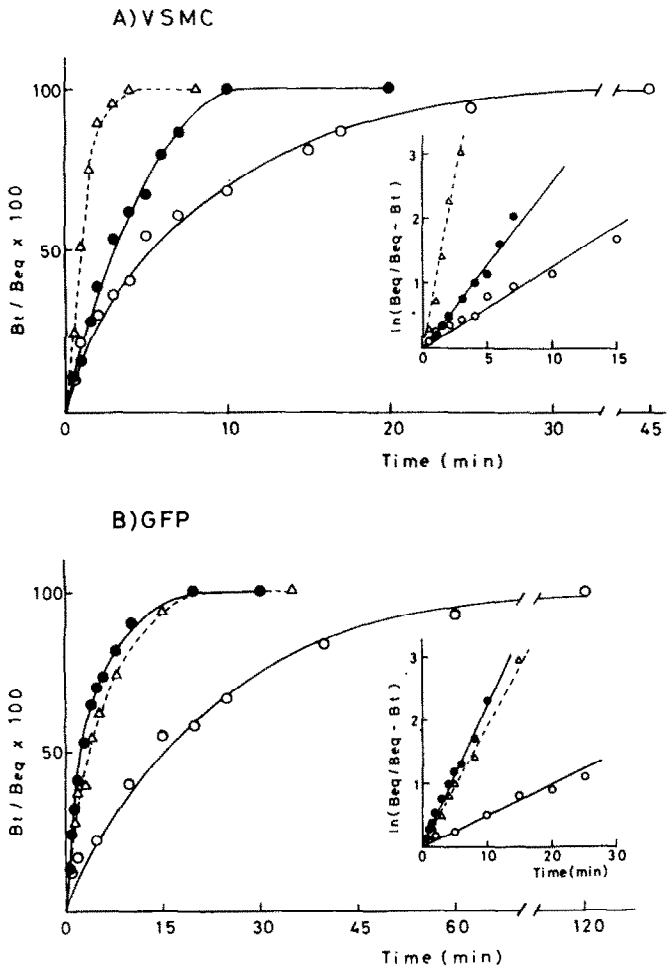

Fig. 1. Time course of association of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to rat VSMC and GFP. (A) Rat VSMC (2×10^6) were incubated with 5.8 nM [^{3}H]S-145 (○), 4.7 nM [^{3}H]SQ29,548 (●) or 23.5 nM [^{3}H]ONO3708 (△) at 24°. Rat GFP (1.7×10^8) were incubated with 2.5 nM [^{3}H]S-145 (○), 2.5 nM [^{3}H]SQ29,548 (●), or 5.2 nM [^{3}H]ONO3708 (△) at 24°. The samples were withdrawn at the indicated times. The specific binding was determined by displacing with a 10 μM concentration of the respective antagonist and expressed with the value of the specific binding at equilibrium at 100%. B_{eq} is the specific binding at equilibrium and B_t is the ^{3}H-labeled antagonists specifically bound at any particular time t. Each point is 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}.

to be 0.120/min for [^{3}H]S-145, 0.235/min for [^{3}H]SQ29,548 and 1.08/min for [^{3}H]ONO3708 for VSMC, and 0.0444/min for [^{3}H]S-145, 0.247/min for [^{3}H]SQ29,548 and 0.197/min for [^{3}H]ONO3708 for GFP. To determine the dissociation rate constant (K_{-1}), VSMC or GFP was incubated with ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to ensure that equilibrium had been reached. At an arbitrary zero time point, the excess unlabeled antagonist (10 μM) was added, and the time course of displacement of each [^{3}H]-radioligand from its binding site was determined for VSMC and GFP. As shown in Fig. 2, dissociation of [^{3}H]S-145 from its specific binding sites was much slower than that of [^{3}H]SQ29,548 or [^{3}H]ONO3708 in both cells, and the time at which one-half of its equilibrium level had been reached was, respectively, about 93 and 116 min in VSMC and GFP. However, the receptor-bound [^{3}H]S-145 in both cells could be partially dissociated by the addition of excess S-145 even after 150 min. This partial dissociation does not imply that [^{3}H]S-145 is irreversibly bound to the TXA_2/PGH_2 receptors, since a complete dissociation of [^{3}H]S-145 was observed after 240 min in both cells by the addition of excess ligand to the incubations at early times (5–10 min) before reaching complete equilibrium (data not shown). In contrast, the dissociation of [^{3}H]SQ29,548 and [^{3}H]ONO3708 from both cells was completed within 90 min. The dissociation pattern of [^{3}H]SQ29,548 was almost the same for VSMC and GFP, whereas that of [^{3}H]ONO3708 in rat VSMC was more rapid than in GFP. Linear transformation of these data (Fig. 2 inset) revealed a K_{-1} value for the binding of each ^{3}H-labeled receptor antagonist to both cells, and we calculated a value for K_1 (association rate constant) according to the

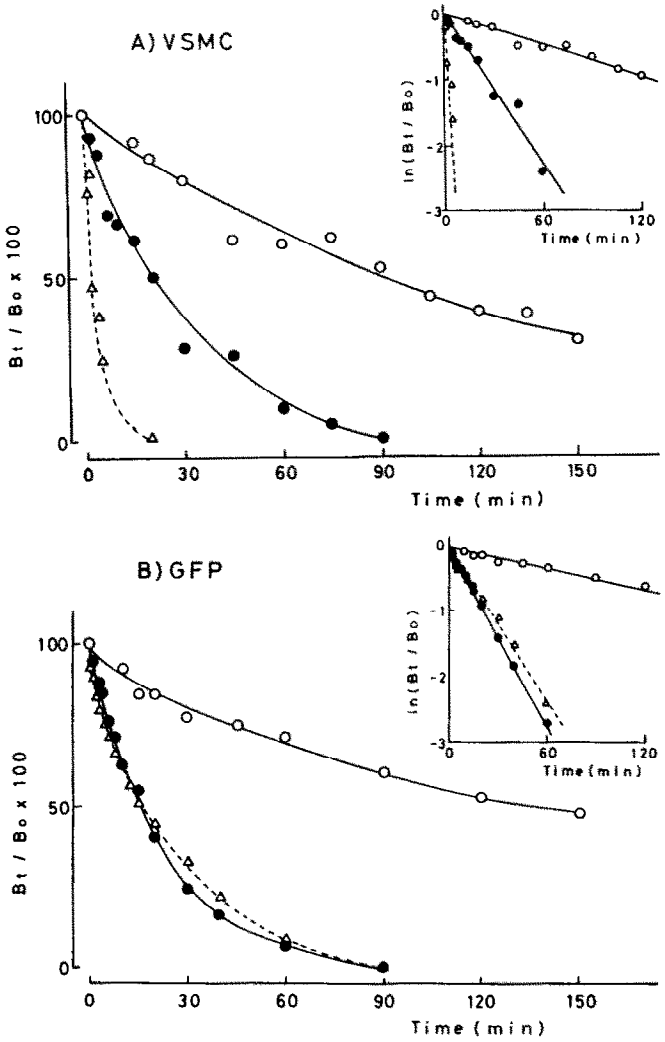

Fig. 2. Time course of dissociation of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists from rat VSMC and GFP. (A) Association of rat VSMC (2×10^6) with 5.8 nM [^{3}H]S-145 (○), 4.7 nM [^{3}H]SQ29,548 (●) or 23.5 nM [^{3}H]ONO3708 (△) was carried out at 24° for, respectively, 60 min, 20 min and 20 min. (B) Association of rat GFP (1.7×10^8) with 2.5 nM [^{3}H]S-145 (○), 2.5 nM [^{3}H]SQ29,548 (●) or 5.2 nM [^{3}H]ONO3708 (△) was carried out at 24° for, respectively, 120 min, 30 min and 30 min. Then, 10 μM unlabeled receptor antagonist was added at time zero to initiate displacement of ^{3}H-labeled antagonist from its specific binding site. The data are expressed with the value of the specific binding at time zero at 100%. B_t is the quantity of ^{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}.

equation $K_1 = (K_{obs}—K_{-1})/[L]$, and also determined the K_d value given by $K_d = K_{-1}/K_1$, all of which are shown in Table 1. The extremely small K_{-1} for [^{3}H]S-145 binding was observed in VSMC as well as in GFP.

Equilibrium binding studies

Studies of saturation binding of [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 to VSMC and GFP revealed that non-specific binding increased linearly with rising concentrations of each [^{3}H]-radioligand, whereas the specific binding exhibited complete saturability in both types of cells. As shown in Fig. 3, Scatchard analysis of these data indicated the existence of a single class of binding sites for each TXA_2/PGH_2 receptor antagonist for both VSMC and GFP, with K_d and B_{max} values as shown in Table 2. The binding density (B_{max}) of each cell was found to be almost the same among the three TXA_2/PGH_2 receptor antagonists. The number of binding sites for [^{3}H]S-145 on rat VSMC was calculated to be *ca.* 4700 receptors per cell, which is approximately 6.5-fold more than that on rat GFP (*ca.* 720 per cell). From comparison with their K_d values, the rank order of binding affinity for rat VSMC was found to be S-145 > SQ29,548 > ONO3708, which is the same order for rat GFP. The K_d value for each receptor antagonist obtained from Scatchard analysis cor-

Table 1. Parameters describing the binding of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to rat VSMC and GFP

Ligand	K_1/M/min	K_{-1}/min	$K_d = K_{-1}/K_1$ (nM)
	(A) VSMC		
[^{3}H]S-145	1.9×10^7	0.0075	0.40
[^{3}H]SQ29,548	4.2×10^7	0.038	0.91
[^{3}H]ONO3708	3.4×10^7	0.28	8.2
	(B) GFP		
[^{3}H]S-145	1.8×10^7	0.0060	0.34
[^{3}H]SQ29,548	8.2×10^7	0.045	0.55
[^{3}H]ONO3708	3.0×10^7	0.040	1.33

The studies were performed and the data analyzed as described in the legends of Figs 1 and 2.

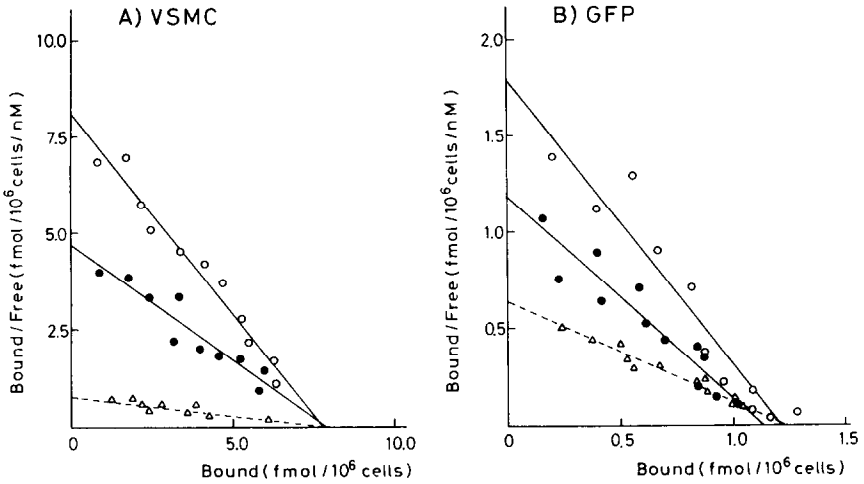

Fig. 3. Scatchard analysis of three ^{3}H-labeled TXA_2/PGH_2 receptor antagonists to rat VSMC and GFP. (A) Rat VSMC (2×10^6) were incubated in the presence of increasing concentrations of [^{3}H]S-145 (○), [^{3}H]SQ29,548 (●) or [^{3}H]ONO3708 (△) at 24° for, respectively, 120 min, 30 min and 30 min. (B) Rat GFP (1.7×10^8) were incubated in the presence of increasing concentrations of [^{3}H]S-145 (○), [^{3}H]SQ29,548 (●) or [^{3}H]ONO3708 (△) at 24° for, respectively, 150 min, 30 min and 30 min. Non-specific binding in the presence of a 10 μM concentration of a respective antagonist was subtracted from each point. Each point is the mean value of triplicate determinations for three experiments.

Table 2. Comparison of K_d and B_{max} values between VSMC and GFP by Scatchard analysis

Ligand	K_d (nM)	B_{max} (fmol/10^6 cells)
	(A) VSMC	
[^{3}H]S-145	0.93 ± 0.20	7.80 ± 0.13
[^{3}H]SQ29,548	1.72 ± 0.21	7.91 ± 0.07
[^{3}H]ONO3708	12.7 ± 2.7	7.98 ± 0.08
	(B) GFP	
[^{3}H]S-145	0.70 ± 0.15	1.20 ± 0.05
[^{3}H]SQ29,548	0.97 ± 0.16	1.15 ± 0.13
[^{3}H]ONO3708	2.01 ± 0.35	1.25 ± 0.07

Data are means ± SE of three experiments. Experiments were performed as described in the legend of Fig. 3.

responded closely with the value of K_d derived from kinetic analysis.

Competitive inhibition of [^{3}H]*S-145 binding to rat VSMC and GFP*

Several compounds were examined for their ability to block the specific binding of [^{3}H]S-145 to VSMC or GFP, and the inhibition curves are shown in Fig. 4. Five TXA_2/PGH_2 receptor antagonists inhibited [^{3}H]S-145 binding to both cells in a concentration-dependent manner, and the potency series of the IC_{50} values was as follows: S-145 ≧ SQ29,548 > ONO3708 > ONO11120 > BM13177. U46619, a TXA_2 mimetic [37], also completely suppressed [^{3}H]S-145 binding, whereas PGD_2, $PGF_{2\alpha}$ and PGE_1 inhibited the ligand binding only at concentrations greater than 1 μM, and complete suppression was not achieved at 100 μM. In addition, TXB_2, a stable metabolite of TXA_2, scarcely competed with the [^{3}H]S-145 binding to rat VSMC and GFP. These results demonstrated that [^{3}H]S-145 became bound to the TXA_2/PGH_2 receptors on both VSMC and

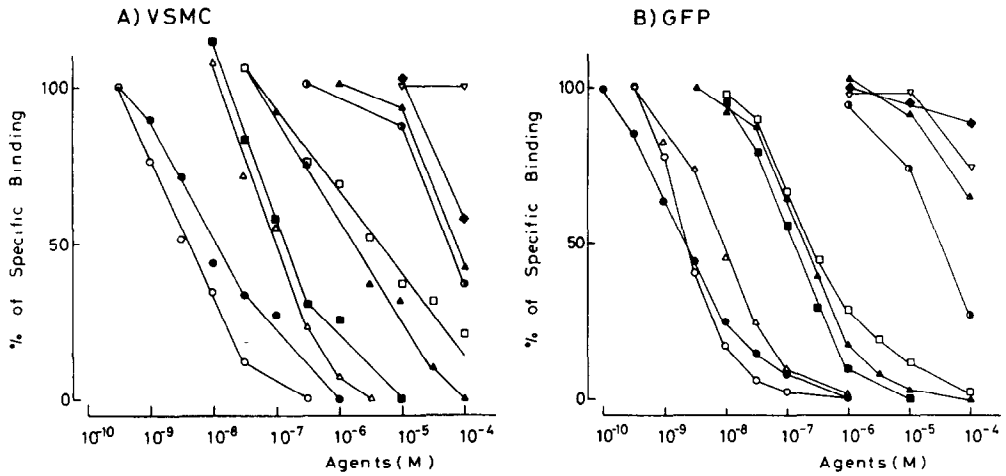

Fig. 4. Competitive inhibition of the specific [^{3}H]S-145 binding to rat VSMC and GFP by various compounds. Rat VSMC (2×10^6) (A) or GFP (1.7×10^8) (B) were incubated, respectively, with 0.5 or 0.7 nM [^{3}H]S-145 in the presence of various concentrations of S-145 (○), SQ29,548 (●), ONO3708 (△), ONO11120 (▲), BM13177 (□), 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 total binding. 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 four independent experimental determinations performed in triplicate.

Table 3. Properties of inhibition of [^{3}H]S-145 binding to rat VSMC and GFP by TXA_2/PGH_2 receptor ligands

Ligands	K_i (nM)	
	VSMC	GFP
S-145	0.77 ± 0.20	1.11 ± 0.32
SQ29,548	2.00 ± 0.30	1.16 ± 0.15
ONO3708	17.2 ± 3.0	4.30 ± 1.50
ONO11120	363.5 ± 54.0	96.1 ± 12.3
BM13177	1530 ± 130	121 ± 22.0
U46619	26.0 ± 3.0	61.6 ± 9.0

K_i values were determined by radioligand competition studies, as described in the legend to Fig. 4, and calculated from the Cheng and Prusoff equations [38] using the K_d values obtained by Scatchard analysis. Data are means ± SE of four experiments.

GFP in a competitive manner. Using the IC_{50} values and the K_d value for [^{3}H]S-145, derived from Scatchard analysis (0.93 nM for VSMC and 0.70 nM for GFP), K_i values for the respective TXA_2/PGH_2 receptor antagonists were approximated using the formula of Cheng and Prusoff [38] (Table 3). The K_i values of unlabeled S-145, SQ29,548 and ONO3708 were almost comparable to the respective K_d values obtained from the saturation studies (Table 2).

Pharmacological specificity for the binding of [^{3}H]*S-145 to VSMC and GFP*

U46619 is known as an extremely potent constrictor of rat aorta [39]. The above five TXA_2/PGH_2 receptor antagonists completely blocked the constriction of rat thoracic aorta induced by 30 nM U46619. The rank order potencies for these antagonists (IC_{50} values), calculated from the normalized concentration–response curves, showed good correlation with the respective K_i values for [^{3}H]S-145 binding to rat VSMC ($r = 0.9797$) (Fig. 5A). However, U46619 can evoke only shape change and not aggregation in rat platelets [40]. We have shown recently the essential role of TXA_2/PGH_2 in mediating the effects of collagen to elicit aggregation of rat platelets [41, 42]. The above five TXA_2/PGH_2 receptor antagonists completely suppressed the collagen-induced aggregation of rat GFP [27], and the negative logarithms of their IC_{50} values against the aggregation correlated well with the corresponding K_i values against [^{3}H]S-145 binding to GFP ($r = 0.9830$) (Fig. 5B). These results indicate that the binding site of [^{3}H]S-145 in both cells is the site responsible for the TXA_2/PGH_2 receptors defined by pharmacological studies.

DISCUSSION

It has been a matter of debate in recent years whether platelet and vascular TXA_2/PGH_2 receptors are identical or not [6, 7]. One of the most important aspects of these comparison studies was the use of platelets and blood vessels from the same species, since there seems to be interspecies differences in the TXA_2/PGH_2 receptors [6, 7]. Recently, Mais *et al.* [7, 8] presented the first pharmacological evidence regarding the existence of different platelet and vessel receptor types in humans as well as dogs. In the present study, we provided the first biochemical approach for the ligand-binding characteristics in rat TXA_2/PGH_2 receptors between platelets and vascular smooth muscle cells by means of radioligand

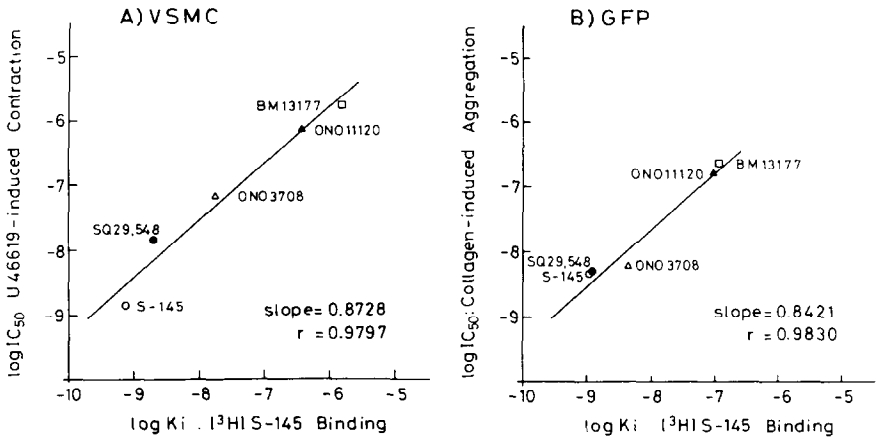

Fig. 5. Graphical correlations of receptor affinity and pharmacological potency of S-145 (○), SQ29,548 (●), ONO3708 (△), ONO11120 (▲) and BM13177 (□) in rat VSMC and GFP. (A) A log-log plot of K_i values ([^{3}H]S-145 binding to rat VSMC) versus IC_{50} (U46619-induced contraction of rat thoracic aorta). The IC_{50} values were taken from the normalized concentration–response curves obtained from the antagonism by the respective antagonists for 30 nM U46619-induced contraction of rat thoracic aorta. The reported points were fitted by least-squares linear regression. (B) A log-log plot of K_i values ([^{3}H]S-145 binding to rat GFP) versus IC_{50} (collagen-induced aggregation of rat GFP). The K_i values and the IC_{50} were taken, respectively, from the results in Table 3 and a previous paper [27], and were analyzed by a linear regression analysis. Each point is the mean value of four experiments performed in triplicate.

binding methods. We utilized cultured VSMC from thoracic aorta and washed platelets (GFP) of the rat as an *in vitro* model, as these cells possess intrinsic TXA_2/PGH_2 receptors corresponding to the pharmacological responses [23, 24].

Different types of TXA_2/PGH_2 receptor antagonists such as [^{3}H]S-145, [^{3}H]SQ29,548 and [^{3}H]ONO3708 were used as receptor radioligands since these compounds have been well characterized as potent and specific antagonists for TXA_2/PGH_2 receptors on rat aorta as well as on rat platelets [23, 27]. The binding of each ^{3}H-labeled ligand displayed high affinity and specificity, as well as saturable and displaceable binding to a single class of recognition sites in both VSMC and GFP. The maximum number of binding sites was virtually identical among the three TXA_2/PGH_2 receptor antagonists in both cells (Table 2). These findings demonstrate that the above three antagonists interact with the same receptor site. This is further supported by the similarity of the K_d values from Scatchard analysis (Table 2) and the K_i values for the respective unlabeled antagonists competing with [^{3}H]S-145 bound on VSMC or GFP (Table 3). Among the ^{3}H-labeled receptor antagonists, S-145 had the highest binding affinity to the TXA_2/PGH_2 receptors in both cells (Fig. 3), which was consistent with the displacement data as described previously [23, 27]. The rank order of binding affinity for the three antagonists was found to be clearly related between the two types of cells; however, the K_d values for SQ29,548 and ONO3708 in VSMC were approximately two and six times larger than that in GFP with the affinity of S-145 being the same for both. Further evidence for the differences of binding affinities came from kinetic analysis of the respective radiolabeled receptor antagonists to VSMC and GFP. No large discrepancies in the kinetic constants (K_1 and K_{-1}) were detected in the S-145 binding to VSMC and GFP, indicating that S-145 has the same affinity for the receptors in both cells. In contrast, the K_1 value for SQ29,548 binding in VSMC was approximately two times smaller than that in GFP, which may explain the differences in K_d values between the two types of cells. As for ONO3708, the K_{-1} value in VSMC was about 7-fold larger than that observed in GFP, while such a large difference was not observed in the K_1 values. Thus, the lower affinity of ONO3708 to VSMC may be due to the more rapid dissociation from the TXA_2/PGH_2 receptors in VSMC, as shown in Fig. 2.

Further investigations were conducted on the inhibitory potencies (K_i) of a series of TXA_2/PGH_2 receptor ligands against [^{3}H]S-145 binding to both types of cells. All of the five TXA_2/PGH_2 receptor antagonists and U46619 completely inhibited [^{3}H]S-145 binding to rat VSMC as well as to GFP (Fig. 4), and the rank order potency of the binding affinity (K_i) of these antagonists correlated well with the K_i values reported for [^{3}H]SQ29,548 binding to VSMC [23] and [^{3}H]U46619 binding to GFP [27]. A good parallelism was also observed between their K_i values against [^{3}H]S-145 binding and the pharmacological activities of both cells, i.e. the inhibitory potencies for the U46619-induced constriction of rat aorta or those for the collagen-induced aggregation of rat platelets (Fig. 5). Thus, it is clear that the recognition sites of S-145 accurately reflect the biochemical and pharmacological specificities of TXA_2/PGH_2 receptors for both VSMC and GFP. The rank orders of inhibitory potencies against the S-145 binding were identical (S-145 > SQ29,548 > ONO3708 > U46619 > ONO11120 > BM13177), indicating the same ligand specificity in the TXA_2/PGH_2 receptors

between GFP and VSMC. However, similar to SQ29,548 and ONO3708, both ONO11120 and BM13177 blocked S-145 binding to GFP more strongly than that to VSMC (Table 3), which explains the pharmacological evidence that these compounds preferentially acted on the platelets rather than on the vascular tissues (Fig. 5). On the other hand, U46619 had more potent inhibitory activity for S-145 binding to VSMC. The ED_{50} value of U46619 to provoke constriction of rat aorta [23] was smaller than that for eliciting aggregation in rat platelets [24], indicating higher affinity of U46619 to VSMC. Thus, these results showed the differences of various TXA_2/PGH_2 receptor ligands in their relative affinity for VSMC and GFP, demonstrating the existence of some heterogeneities in the ligand binding sites of the TXA_2/PGH_2 receptors in both cells.

Recently, Mais and coworkers [7, 8] provided the pharmacological evidence for the presence of the different classes of TXA_2/PGH_2 receptors by comparing the structure–activity relationships for a series of 13-azapinane TXA_2/PGH_2 receptor antagonists for human and canine platelets and saphenous veins. Four of 13-azapinane TXA_2 analogs were antagonists in the platelets but possessed agonist activity in the vessels [8], the same as with carbocyclic TXA_2 [11]. Thus, they named the platelet receptor $[TXA_2/PGH_2)_\alpha$ (α for aggregation) and the vessel receptor $[TXA_2/PGH_2]_\tau$ (τ for tone) [7, 8]. However, the pharmacological approaches carry some limitations as to the interpretation of the results because of differences in the ligand diffusion, uptake and/or metabolism. The radioligand binding assays used in the present study can thus provide a direct means for examining the interaction of the ligand and receptors. Since the rank orders of binding affinity of several ligands were identical in rat VSMC and GFP, there seems to be the same type of TXA_2/PGH_2 receptors in both cells. However, distinct differences were observed in the absolute K_d or K_i values for the respective compounds between the two cells, suggesting the existence of some differences in the ligand–receptor interaction between VSMC and GFP. These differences on the basis of kinetic study may be attributed to differences in the accessibility of the ligands to the receptor in two cell types, since this is an important factor when dealing with ligands of widely different structures [7].

We provided here the first biochemical comparison of the TXA_2/PGH_2 receptors for platelets and vascular smooth muscle cells. The TXA_2/PGH_2 receptors in the two cells may possess similar specificities as well as some differences in the affinity for various receptor ligands, although the exact nature remains to be clarified. The platelet TXA_2/PGH_2 receptors have been solubilized in active form and characterized by hydroxynamic studies [21, 22]. The availability of cultured VSMC as an *in vitro* model and [^{3}H]S-145 as a highly specific radioligand may thus facilitate further investigations into the biochemical nature of the TXA_2/PGH_2 receptors in the blood vessels.

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