Biochemical Pharmacology, Vol. 42, No. 3, pp. 537–544, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

PLATELET AND VASCULAR THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS

EVIDENCE FOR DIFFERENT SUBCLASSES IN THE RAT

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(*Received* 27 *August* 1990; *accepted* 20 *February* 1991)

Abstract—Thromboxane A_2 (TXA_2) and its precursor prostaglandin H_2 (PGH_2) induce platelet aggregation and vascular contraction through shared cell surface receptors commonly referred to as TXA_2 or TXA_2/PGH_2 receptors. Whether different subclasses of TXA_2/PGH_2 receptors exist in platelets and vascular smooth muscle cells is controversial. In this study, TXA_2 receptors on washed rat and human platelets and cultured rat aortic smooth muscle cells (RASMC) were characterized using radioligand competition binding assays with the ^{125}I-labeled TXA_2/PGH_2 receptor agonist [1*S*-(1α,2β(5Z),3α(1E,3R*),4α)] -7- [3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl) -7- oxabicyclo-[2.2.1]-heptan-2-yl] -5- heptenoic acid (I-BOP) and various agonists and antagonists. Scatchard analyses of equilibrium binding data revealed K_d values of 205 ± 68 pM (N = 6), 2.2 ± 0.3 nM (N = 9) and 310 ± 60 pM (N = 7) and B_{max} values of 1.3 ± 0.45 fmol/10^6 platelets, 2.8 ± 0.2 fmol/10^6 platelets and 20.9 ± 2.2 fmol/10^6 cells for rat and human platelets and RASMC, respectively. Concentration-dependent increases in intracellular free Ca^{2+} concentrations induced by I-BOP were observed in RASMC loaded with the calcium sensitive dye fura-2. The IC_{50} values for various TXA_2/PGH_2 analogues in competition binding assays with ^{125}I-BOP were determined. Based on their IC_{50} values, the rank orders were I-BOP < L657925 < ONO11113 ≤ SQ29548 < PTA-TPO < PTA-NO ≤ L657926 ≤ I-PTA-OH < PTA-OH[2] = meta-I-PTA-PO ≤ ONO11120[2] = ONO11120[1] < PTA-OH[1] in rat platelets, I-BOP < SQ29548 < PTA-TPO = L657925 ≤ ONO11113 < I-PTA-OH < PTA-NO ≤ meta-I-PTA-PO ≤ PTA-OH[2] < ONO11120[2] ≤ ONO11120[1] < L657926 ≤ PTA-OH[1] in human platelets, and I-BOP < L657925 < ONO11113 ≤ SQ29548 < ONO11120[2] ≤ L657926 ≤ PTA-OH[2] < PTA-TPO < ONO11120[1] < I-PTA-OH < meta-I-PTA-PO < PTA-NO < PTA-OH[1] in RASMC. For the structurally dissimilar compounds I-BOP, SQ29548, ONO-11113, L657925 and L657926, there were no significant differences in the rank order potencies between the three tissues. In contrast, the rank order potencies for the 13-azapinane TXA_2 analogs were significantly different in rat platelets compared to RASMC, and human platelets compared to RASMC, but not significantly different between rat and human platelets. The ratios of IC_{50} values for the two sets of epimeric pairs of 13-azapinane TXA_2 analogs (ONO11120[1] and [2] and PTA-OH[1] and [2]) were significantly different ($P < 0.05$) for rat and human platelets compared to RASMC but not different between rat and human platelets. The results are consistent with the notion that TXA_2/PGH_2 receptors on platelets are different from those on RASMC, and 13-azapinane TXA_2 analogs may be useful compounds for discriminating between the two classes of TXA_2/PGH_2 receptors.

Thromboxane A_2 (TXA_2)‡ and its precursor prostaglandin H_2 (PGH_2) are potent vasoconstrictors and proaggregatory substances [1–3] that act through a putative common membrane receptor (TXA_2/PGH_2) [4].

The presence of TXA_2/PGH_2 receptors on

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‡ Abbreviations: TXA_2: thromboxane A_2; PGH_2: prostaglandin H_2; I-BOP: [1*S*-(1α,2β(5Z),3α(1E,3R*),-4α)]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid; L657925(−) and L657926(+): (−)- and (+)-9-chlorobenzyl-6-fluoro-1,2,3,4-tetrahydrocarbazol-1-yl acetic acid; ONO11113: 9,11-epithio-11,12-methano-TXA_2; SQ29548: [1*S*-(1α,2β-(5Z),3β,4α)]-7-{3-[[2-[(phenylamino)carbonyl]hydrazino] - methyl] - 7 - oxabicyclo-[2.2.1]hept-2-yl}-5-heptenoic acid; PTA-TPO: 9,11-dimethylmethano-11,12-methano-16-thiophenoxy-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranorthromboxane A_2; PTA-NO: 9,11-dimethylmethano-11,12-methano-16-(2-naphthoxy)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranorthromboxane A_2; I-PTA-OH: 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranorthromboxane A_2; PTA-OH: 9,11-dimethyl-methano-11,12-methano-16-(*p*-hydroxyphenyl)-13,14-dihydro-13-aza-15(α or β)-ω-tetranorthromboxane A_2; Meta-I-PTA-PO: 9,11-dimethylmethano-11,12-methano-16-(*m*-iodophenoxy)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranorthromboxane A_2; ONO11120: 9,11-dimethylmethano-11,12-methano-16-(phenyl)-13,14-dihydro-13-aza-15(α or β)-ω-tetranorthromboxane A_2; RASMC: rat aorta smooth muscle cells; WRP: washed rat platelets; WHP: washed human platelets; B_{max}: maximum binding; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate-buffered saline; Tris: tris(hydroxymethyl) aminomethane; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; and EGTA: ethyleneglycolbis(aminoethylether)tetra-acetate.

platelets and blood vessels has been established using both pharmacologic [4, 5] and radioligand binding studies [6–8]. Controversy has arisen as to whether or not platelet and vascular TXA_2/PGH_2 receptors are different. Several pharmacological studies using human platelets and blood vessels from different species supported the notion that they were different [2, 9]. However, since these studies crossed species and often used only a single compound, the possibility of species difference versus true receptor heterogeneity remained uncertain. Mais *et al.* [10–12] using analogues of 13-azapinane TXA_2 provided pharmacological evidence that in the human and dog, the platelet and vascular TXA_2/PGH_2 receptors were different. They named the platelet receptor $(TXA_2/PGH_2)_\alpha$, α for aggregation, and the vascular receptor $(TXA_2/PGH_2)_\tau$, τ for tone. Morinelli *et al.* [13] also provided pharmacological evidence that platelet and vascular receptors were different, using analogs of 10,10-difluoro-TXA_2. However, other pharmacologic and radioligand binding studies have failed to find differences between the platelet and vascular receptors even when crossing species [14–16]. Thus, it remains controversial whether platelet and vascular TXA_2/PGH_2 receptors are different.

The current study was designed to test the hypothesis that the platelet and vascular TXA_2/PGH_2 receptors are different. Specifically, the rank order potency for a series of TXA_2/PGH_2 receptor agonists and antagonists to compete for binding with ^{125}I-BOP ([1*S*-(1α,2β(5Z),3α(1E,3R*),4α)]-7-{3- (3-hydroxy- 4-(4'-iodophenoxy)- 1-butenyl)- 7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid) in cultured rat aortic smooth muscle cells and washed rat and human platelets was determined.

MATERIALS AND METHODS

Materials. I-BOP, ^{125}I-BOP and the 13-azapinane TXA_2 analogues were synthesized in our laboratory as previously described [12, 17]. The structures of all the compounds used in these studies are shown in Fig. 1. The epimers of ONO11120 and PTA-OH were separated by HPLC as previously reported [11]. The following were gifts: ONO11113 and ONO11120 (ONO Pharmaceutical Co., Osaka, Japan); L657925 and L657926 (Merck Frosst Canada, Inc., Point Claire-Dorval, Quebec, Canada) and SQ29548 (Dr. Martin Ogletree, Squibb Institute for Medical Research, Princeton, NJ, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, antibiotic–antimycotic mixture (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, U.S.A.). All other reagents and supplies were of the highest purity available from Fisher Scientific (Houston, TX, U.S.A.), or Sigma (St. Louis, MO, U.S.A.).

Preparation of washed rat and human platelets. Blood was drawn into a syringe containing indomethacin (10 μM) and EDTA (5 mM) (final concentrations) via cardiac puncture from male, Long-Evans rats after anesthesia with sodium pentobarbital (30 mg/kg). Human blood was drawn into a syringe containing indomethacin (10 μM) and EDTA (5 mM) via venipuncture from normal volunteers. the blood was centrifuged at 100 *g* for rat blood or at 132 *g* for human blood for 20 min at room temperature. The resulting platelet-rich plasma was centrifuged at 1280 *g* for 20 min at room temperature and the platelet pellet was resuspended in Tris-buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM glucose and 10 μM indomethacin, pH 7.4, at 37°) to a final concentration of 1×10^8 platelets/mL for binding assays.

Culture and preparation of rat aortic smooth muscle cells. Rat aortic smooth muscle cells were cultured using a modification of the procedure of Ross [18]. Briefly, male Long-Evans rats were anesthetized using sodium pentobarbital, and the aorta was removed and placed into sterile DMEM. The aorta was opened longitudinally and dissected into 2- to 3-mm segments and placed intima side down on a plastic petri dish. These segments were incubated in 10 mL of DMEM containing 10% (v/v) FBS and 1% antibiotic–antimycotic solution at 37° in a humidified atmosphere of 95% air/5% CO_2. The growth medium was changed every 3–4 days. Upon reaching confluence, cells were harvested using 3 mL of 0.05% trypsin-EDTA. The suspension was then dispensed into flasks for further growth.

After reaching confluence, the medium was changed and 24 hr later the cells were harvested for the binding assays. The cells were washed twice with phosphate-buffered saline (pH 7.4, 37°) containing 10 μM indomethacin. The cells were harvested using exposure to 3 mL of 0.05% trypsin-EDTA for 1–2 min. The cells were suspended into 30 mL of DMEM containing 10% FBS and centrifuged at 100 *g* for 5 min, and then washed once with 20 mL of Hanks' balanced salt solution (135 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 0.45 mM KH_2PO_4, 0.8 mM $MgSO_4$, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 5.5 mM glucose). The washed cells were centrifuged again at 100 *g* for 5 min, resuspended in the same buffer, and then used in the radioligand binding assay. Cells were used between passages 3 and 8. RASMC maintained their binding characteristics for I-BOP without change through this passage number. Cell viability was determined using trypan blue exclusion and was ~95%.

Radioligand binding experiments. The radioligand binding assays were performed by incubating cells (platelets: 1×10^7 platelets in a final volume of 200 μL; RASMC: 5×10^5 cells in a final volume of 400 μL) with ~2×10^4 cpm (~40 pM) of ^{125}I-BOP and competing ligands in Tris/saline buffer containing 10 μM indomethacin. After incubation for 30 min at 37°, the reaction was stopped by adding 4 mL of ice-cold buffer and the mixture was filtered through Whatman GF/C filters and washed three times with 4 mL of ice-cold wash buffer within 10 sec. Non-specific binding was defined as the amount of bound radioactivity in the presence of L657925 (10 μM), a stereoselective TXA_2/PGH_2 receptor antagonist [19]. Specific binding of ^{125}I-BOP was ~90% in the platelets and ~60% in the RASMC. K_d and B_{max} values for I-BOP were determined using the LIGAND computer program [20]. The IC_{50} values for other compounds were obtained from log-logit linear regression analyses.

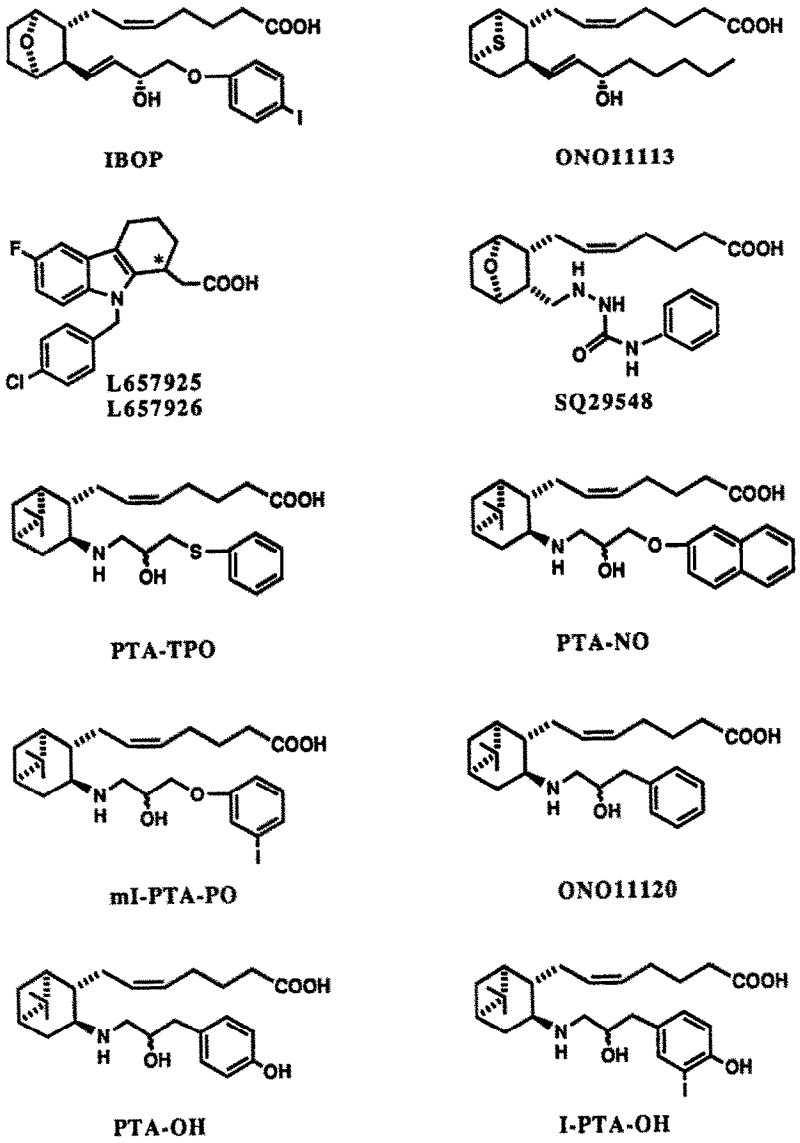

Fig. 1. Structures of thromboxane A_2 analogues used in this study.

Measurement of intracellular free calcium concentrations. RASMC were harvested as previously described and resuspended in HEPES buffer (10 mM HEPES, 145 mM NaCl, 5.0 mM KCl, 5.5 mM glucose, pH 7.4) without Ca^{2+} and Mg^{2+} and incubated with 8 μM fura-2 AM for 1 hr at 37°. After two washes with the buffer, cells were resuspended to 2×10^5 cells/mL in HEPES buffer containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. The cells were placed in quartz cuvettes at 37° with continuous stirring. Basal and I-BOP stimulated intracellular free Ca^{2+} concentrations were measured using a fluorescence spectrophotometer (Aminco-Bowman, Silver Spring, MD, U.S.A.) with excitation and emission wavelengths of 340 and 500 nm, respectively. Maximum fluorescence (F_{max}) was defined by lysing the cells with 10 mM digitonin and the minimum fluorescence (F_{min}) by the subsequent addition of 10 mM EGTA and 20 mM Tris base (pH 8). The intracellular free calcium concentrations were determined from the observed fluorescence (F_{obs}), F_{max} and F_{min} using the following formula [21]:

$$[Ca^{2+}]_i = (F_{obs} - F_{min})/(F_{max} - F_{obs}) \times 224\ nM$$

The EC_{50} values were obtained from a log-logit linear regression analysis.

Statistics. All data are expressed as mean ± SEM. Student's unpaired *t*-test was employed to determine the differences between the IC_{50} values of the pairs of epimers. Spearman's rank order test was used for determining if differences existed between rank orders in the various cell types. A P value of < 0.05 signified that the rank order potencies for the compounds in platelets and blood vessels were not significantly different from each other.

RESULTS

Binding studies in washed rat platelets. Binding of ^{125}I-BOP to washed rat platelets was saturable and displaceable. Scatchard analysis of the equilibrium binding data revealed a single class of receptors ($K_d = 205 \pm 68$ pM; $B_{max} = 1.3 \pm 0.45$ fmol/10^6 platelets or 771 ± 271 sites/platelet, N = 6). A series of TXA_2/PGH_2 receptor agonists and antagonists were used to further characterize the binding of ^{125}I-BOP to the receptor. In competition binding assays with ^{125}I-BOP, the rank order potency for the compounds based on their IC_{50} values was: I-BOP < L657925 < ONO11113 ≤ SQ29548 < PTA-TPO < PTA-NO ≤ L657926 ≤ I-PTA-OH < PTA-OH[2] = meta-I-PTA-PO ≤ ONO11120[2] = ONO11120[1] < PTA-OH[1] (Table 1). Stereoselectivity of binding was observed using the stereoisomers, L657925 and L657926 (Table 1) which had IC_{50} values of 3.2 ± 1.1 nM (N = 4) and 230 ± 52 nM (N = 4), respectively. The epimers ONO11120[1] and ONO11120[2] showed no significant differences in binding affinities to washed rat platelets (Table 1, Fig. 2.). In contrast, the affinities of PTA-OH[1] and PTA-OH[2] in washed rat platelets differed by a factor of approximately 5 ($P < 0.05$) (Table 1, Fig. 2).

Binding studies in washed human platelets. I-BOP has been shown previously to bind to washed human platelets with a K_d value of 2.2 ± 0.3 nM and a B_{max} or 2.8 ± 0.2 fmol/10^6 platelets or 1699 ± 162 sites/platelet [17]. Competition binding assays performed in this study revealed the following rank order potency for the compounds based on their IC_{50} values: I-BOP < SQ29548 < PTA-TPO = L657925 ≤ ONO11113 < I-PTA-OH < PTA-NO ≤ meta-I-PTA-PO ≤ PTA-OH[2] < ONO11120[2] ≤ ONO11120[1] < L657926 ≤ PTA-OH[1] (Table 1). In washed human platelets, the epimers PTA-OH[1] and PTA-OH[2] showed significantly different ($P < 0.05$) IC_{50} values in competition binding assays, whereas the epimers ONO11120[1] and ONO11120[2] did not (Table 1, Fig. 2).

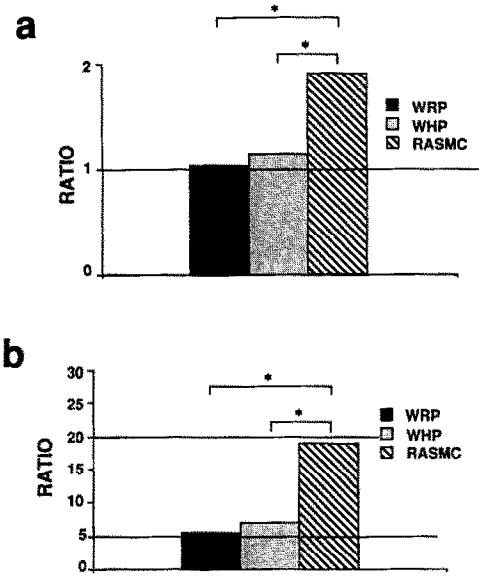

Fig. 2. Ratios of potencies of epimeric pairs on ONO11120 and PTA-OH in washed rat platelets (WRP), rat aorta smooth muscle cells (RASMC), and washed human platelets (WHP). Numbers indicated on the *X* axis are the IC_{50} values of epimer 1 divided by the IC_{50} value of epimer 2. Panel a is the epimeric pair of ONO11120, and panel b is the epimeric pair of PTA-OH. Key: (*) $P < 0.05$.

Binding studies in cultured rat aorta smooth muscle cells. ^{125}I-BOP binding was saturable and displaceable in RASMC. Scatchard analysis of the equilibrium binding data revealed a single class of binding sites ($K_d = 310 \pm 60$ pM; $B_{max} = 20.9 \pm 2.2$ fmol/10^6 cells or $10,872 \pm 3,244$ receptors/cell, N = 7) (Fig. 3). The IC_{50} values for the series of compounds in the binding assays showed the following rank order potency: I-BOP < L657925 < ONO11113 ≤ SQ29548 < ONO11120[2] ≤ L657926 ≤ PTA-OH[2] < PTA-TPO < ONO11120[1] < I-PTA-OH

Table 1. IC_{50} Values of various TXA_2/PGH_2 analogs for competing with ^{125}I-BOP binding to washed rat platelets, rat aorta smooth muscle cells and washed human platelets

	IC_{50} (nM)		
	Washed rat platelets	Rat aorta smooth muscle cells	Washed human platelets
I-BOP	0.2 ± 0.1 (6) [1]	0.3 ± 0.1 (7) [1]	2.2 ± 0.3* [1]
L657925	3.2 ± 1.1 (4) [2]	4.6 ± 1.0 (5) [2]	15.4 ± 4.0* [4]
ONO11113	7.2 ± 2.8 (4) [3]	7.4 ± 1.8 (5) [3]	17.0 ± 1.8* [5]
SQ29548	8.1 ± 1.1 (5) [4]	10.8 ± 2.4 (5) [4]	4.7 ± 0.5* [2]
PTA-TPO	82 ± 5.1 (3) [5]	509 ± 63 (4) [8]	15.1 ± 4.7 (3) [3]
PTA-NO	213 ± 43 (3) [6]	1606 ± 348 (3) [12]	49.2 ± 11 (3) [7]
L657926	230 ± 52 (4) [7]	329 ± 56 (6) [6]	424 ± 90* [12]
I-PTA-OH	260 ± 37 (4) [8]	638 ± 97 (4) [10]	21.0 ± 5.0† [6]
PTA-OH[2]	345 ± 71 (3) [9]	343 ± 54 (4) [7]	66 ± 5 (5) [9]
meta-I-PTA-PO	349 ± 52 (4) [10]	1227 ± 93 (4) [11]	60 ± 11 (3) [8]
ONO11120[2]	370 ± 7 (3) [11]	313 ± 46 (3) [5]	124 ± 3 (3) [10]
ONO11120[1]	383 ± 28 (3) [12]	596 ± 25 (3) [9]	142 ± 7 (3) [11]
PTA-OH[1]	1920 ± 122 (3) [13]	6528 ± 604 (3) [13]	464 ± 98 (5) [13]

All data are means ± SEM; numbers in parentheses = number of experiments. Numbers in brackets = rank for the respective tissue.

* Ref. 17.

† Ref. 7.

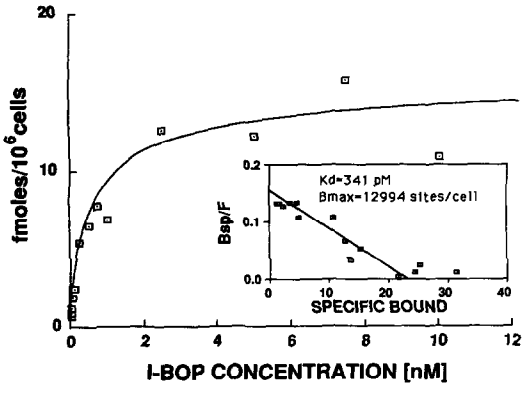

Fig. 3. Representative equilibrium binding analysis of I-BOP to cultured rat aorta smooth muscle cells. The assay conditions are described in Materials and Methods. Insert: Scatchard analysis of saturation binding data. Specific bound/free (Bsp/F) is shown as a function of specific bound.

< meta-I-PTA-PO < PTA-NO < PTA-OH[1] (Table 1). The stereoisomers L657925 and L657926 showed a 72-fold difference in IC_{50} values in washed rat platelets and RASMC and a 28-fold difference in washed human platelets (N = 6) (Table 1). In RASMC, the epimers, ONO11120[1] and ONO11120[2] and PTA-OH[1] and PTA-OH[2], showed 2- and 19-fold differences ($P < 0.01$) in binding affinities (Table 1, Fig. 2).

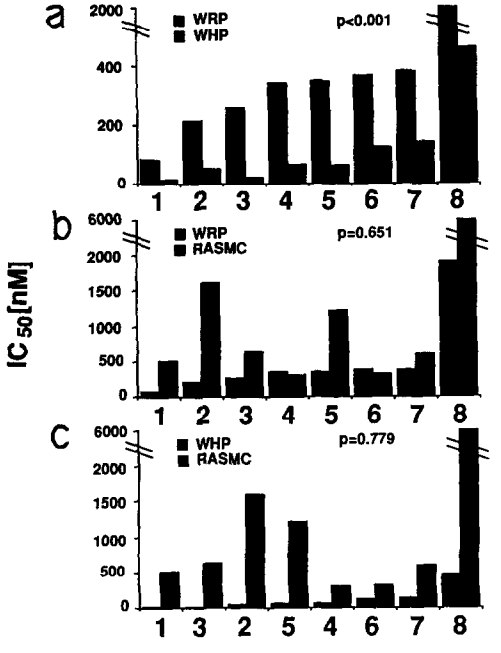

Fig. 4. Comparison of rank order potencies for 13-azapinane T analogues in WRP, RASMC and WHP. Spearman's rank order test was used for comparing the rank orders. Compounds shown by numbers are as follows: 1, PTA-TPO: 2, PTA-NO; 3, PTA-OH; 4, PTA-OH(2); 5, meta I-PTA-PO; 6, ONO11120(2); 7, ONO11120(1); and 8, PTA-OH(1). $P < 0.05$ signifies that the rank orders are not different from each other.

Comparison of rank order potencies. Comparison of the rank order potencies among washed rat and human platelets and RASMC for all 13 compounds tested showed that they were not significantly different. However, a significant difference between the rank order potencies in rat platelets and RASMC was observed when only the eight 13-azapinane compounds were compared (Fig. 4). There was also a significant difference between the rank order potencies in the washed human platelets and that of RASMC. However, the rank order potencies of the compounds in washed rat platelets and human platelets were not different from each other even when only the eight 13-azapinane TXA_2 compounds were analyzed (Fig. 4A). The potency ratios for the epimers ONO11120[1] and [2] and PTA-OH[1] and [2] were significantly greater in RASMC compared to either rat or washed human platelets (Fig. 2, a and b). All eight 13-azapinane TXA_2 compounds were more potent in the washed human platelets than in the washed rat platelets and RASMC. On the other hand, I-BOP, ONO11113, L657925 and L657926 were less potent in washed human platelets. Furthermore, although ^{125}I-PTA-OH, a TXA_2/PGH_2 receptor antagonist, binds to washed human platelets [7], it showed no specific binding in both washed rat platelets and RASMC (data not shown).

Measurement of intracellular free calcium concentration. To confirm that I-BOP bound to a functional TXA_2 receptor in RASMC, we assessed its effect on intracellular free Ca^{2+}. I-BOP produced a concentration-dependent increase in fura-2 fluorescence in suspensions of RASMC. At concentrations over 500 nM, I-BOP caused the maximum increase in intracellular free calcium (61 ± 6.6 nM). The EC_{50} value for I-BOP was 20.2 ± 2.1 nM (N = 5) (Fig. 5). The responses to 1 μM I-BOP were antagonized by two different TXA_2/PGH_2 receptor antagonists, L657925 and SQ29548, at the concentration of 1 μM each (data not shown).

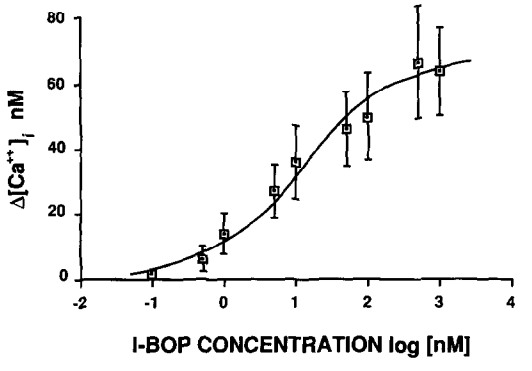

Fig. 5. Concentration-dependent increases in intracellular free Ca^{2+} concentration induced by I-BOP. Cells loaded with fura-2 AM were resuspended in HEPES buffer and stimulated by various concentrations of I-BOP. The EC_{50} value was 20.2 ± 2.1 nM. Values are means ± SEM (N = 5).

DISCUSSION

This study describes the binding of ^{125}I-BOP to TXA_2/PGH_2 receptors in rat platelets, cultured rat aortic vascular smooth muscle cells, and washed human platelets and its competition with a series of two agonists, eleven antagonists and a pair of stereoisomers. These observations strongly support the notion that I-BOP was binding to TXA_2/PGH_2 receptors in all three cell types.

The previously reported radiolabeled agonists [^{3}H]U46619 [22, 23] and [^{3}H]U44069 [24] are less potent (K_d values of 109 and 70 nM, respectively) in washed human platelets and have lower specific binding (40%) [22–24] compared to I-BOP in platelets. I-BOP is the first ^{125}I-radiolabeled TXA_2/PGH_2 receptor agonist and has a high affinity and specific binding. I-BOP bound to a single class of receptors with K_d values of 205 and 310 pM for washed rat platelets and RASMC, respectively, making it the most potent radiolabeled TXA_2 analogue yet reported in the recently reported ^{3}H-labeled antagonists S-145 [25] and SQ29548 [8]. I-BOP binds to a single class of binding sites in human platelets with a K_d of 2.2 nM [17]. However, Dorn [26] reported two binding sites, one with a K_d of approximately 200 pM and the other of approximately 2 nM. We have also found recently two binding sites for I-BOP with similar K_d values when binding is performed at pH 6.5 (unpublished observations). Thus, the higher affinity site in human platelets identified by Dorn [26] and our group may be comparable to the site identified by I-BOP in the RASMC. However, this remains to be determined.

Previous pharmacologic studies have demonstrated that TXA_2 mimetics increased $[Ca^{2+}]_i$ in RASMC [27]. To verify that I-BOP bound to TXA_2/PGH_2 receptors in RASMC, its ability to increase intracellular free Ca^{2+} concentrations was determined. I-BOP increased $[Ca^{2+}]_i$ in a concentration-dependent manner and was antagonized by two TXA_2/PGH_2 antagonists SQ29548 and L657925. These results indicate that the receptors recognized by I-BOP are coupled to a second messenger system that results in an increase in $[Ca^{2+}]_i$. However, the K_d value of I-BOP was 310 pM in the binding assay in RASMC, while it had an EC_{50} value of 20.2 nM to evoke an increase in $[Ca^{2+}]_i$. The reason for the discrepancy between K_d and IC_{50} values is unclear. Previous studies of the effects of TXA_2 mimetics to increase $[Ca^{2+}]_i$ concentrations in cultured rat [27] and human [28, 29] vascular smooth muscle cells have also required large concentrations of the agonists, higher than their apparent affinity for the receptor. Thus, the possibility exists that the TXA_2/PGH_2 receptor in cultured vascular smooth muscle cells is poorly coupled to this second messenger system or that these are not the optimal conditions for the measurement of changes in intracellular free calcium concentration.

To determine if subclasses of TXA_2/PGH_2 receptors exist in platelets and vascular smooth muscle cells, correlations between the rank order potencies of various TXA_2/PGH_2 analogues in washed rat platelets and RASMC were analyzed using Spearman's rank order test. There was a significant correlation between the rank order potencies of the 13 TXA_2/PGH_2 analogues among these different cell types raising the possibility that rat platelets and RASMC possess the same class of TXA_2/PGH_2 receptors. These results are similar to those of Swayne *et al.* [15] who reported that the rank order potencies for a series of non-13-azapinane TXA_2 analogues were not different for platelet and vascular receptors.

In contrast to these results, analysis of the data for the 13-azapinane compounds alone showed that the rank order potencies of these compounds were significantly different between washed rat platelets and RASMC and between washed human platelets and RASMC. In contrast, the rank order potencies for the eight 13-azapinane TXA_2 compounds in washed rat platelets and washed human platelets were not different from each other. The potency ratios for the epimers of the 13-azapinane TXA_2 analogues, ONO11120 and PTA-OH, were significantly greater in the RASMC compared to washed rat platelets. Mais *et al.* [11] also reported that the individual epimers were equipotent in human platelets but for each of the pairs one was three times more potent than the other in antagonizing TXA_2 mimetic-induced contraction of canine saphenous veins. These results suggest that the vascular TXA_2/PGH_2 receptor can distinguish the stereochemistry of the 15-hydroxy group of the 13-azapinane analogues but not the platelet receptor. All the 13-azapinane TXA_2 analogues had higher IC_{50} values in RASMC compared to washed human platelets and washed rat platelets except for PTA-OH[2] and ONO11120[2]. This observation is similar to that reported by Dorn *et al.* [28], who showed that I-PTA-OH was much less potent in cultured human saphenous vein smooth muscle cells compared to human platelets. Thus, it would appear that 13-azapinane TXA_2 analogues possess the ability to discriminate between platelet and vascular TXA_2/PGH_2 receptors whereas non-13-azapinane TXA_2 analogues do not. This observation is analogous to that which has been reported for other receptors, that is, some ligands for the receptors discriminated between subtypes whereas others did not. Collectively, the data strongly support the existence of different subclasses of TXA_2/PGH_2 receptors in rat and human platelets compared to RASMC. It remains to be determined whether the pinane nucleus and/or the 13-aza group of these analogues is the discriminating pharmacophore.

All the 13-azapinane TXA_2 analogues showed lower IC_{50} values in washed human platelets compared to washed rat platelets but the non-13-azapinane TXA_2 analogues I-BOP, ONO11113, L657925 and L657926 showed the opposite results. Thus, it is still uncertain if there are species differences in the TXA_2/PGH_2 receptors in rat platelets compared to human platelets. Indeed, it is well known that rat platelets do not aggregate to TXA_2 mimetics, yet require endogenous metabolism of arachidonic acid to labile aggregation stimulating substances for aggregation to occur [30]. Thus, it is uncertain at the present time if rat and human platelet TXA_2/PGH_2 receptors are the same or may be different. Clearly, additional studies are required to answer this question.

In conclusion, both rat platelets and RASMC have a single class of TXA_2/PGH_2 receptors recognized by I-BOP and the competition binding assay results support the existence of different subclasses of TXA_2/PGH_2 receptors in these two different cell types from the same species. Clearly, the ultimate proof for the presence of subclasses of receptors will reside in the purification and/or cloning of the receptor from these sources.

Acknowledgements—Supported in part by NIH Grant HL36838 and the Ciba-Geigy Pharmaceutical Co. This work was done during the tenure of a Grant-in-Aid from the American Heart Association and Bristol Meyers. The authors are grateful to Ms. J. Nelson for typing assistance.

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