Biochemical Pharmacology, Vol. 43, No. 8, pp. 1747–1752, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

CHARACTERIZATION OF THE THROMBOXANE RECEPTOR MEDIATING PROSTACYCLIN RELEASE FROM CULTURED ENDOTHELIAL CELLS

JENNIFER A. HUNT, JANET E. MERRITT,* JOHN MACDERMOT† and MARY KEEN‡

Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TT; *SmithKline Beecham, The Frythe, Welwyn, Hertfordshire AL6 9AR and †Department of Clinical Pharmacology, The Royal Postgraduate Medical School, Hammersmith Hospital, Ducane Road, London W12 0NN, U.K.

(*Received* 24 *October* 1991; *accepted* 14 *January* 1992)

Abstract—The thromboxane A_2 (TXA_2) mimetic, 9,11-dideoxy-11,9-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), mobilized calcium in the bovine aortic endothelial cell line AG4762 and stimulated release of prostacyclin from these cells. The U46619-stimulated release of prostacyclin could be inhibited by TXA_2 antagonists with the order of potency [ls-[1<a, 2<b(5z), 3<b, 4<a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548) > 4-[2-(4-chlorobenzene-sulphonamido)ethyl]phenylacetic acid (BM13505) > 4-[2-(phenylsulphonamido)-ethyl]phenoxyacetic acid (BM13177), which was consistent with release being mediated by a TXA_2 (TP) receptor. The TP receptor ligands, [^{3}H]SQ29548 and 9,11-dimethylmethano-16(3-[^{125}I]iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15-ω-o-tetranor-thromboxane ([^{125}I]-PTA-OH), both appeared to bind to a homogenous population of sites in AG4762 cell membranes. The affinities of [^{3}H]SQ29548 and [^{125}I]PTA-OH were $\approx$ 10 nM and $\approx$ 0.3 nM, respectively, and the density of sites labelled by either ligand was $\approx$ 25 fmol/mg protein. Under conditions where equilibrium was approached, the specific binding of [^{3}H]SQ29548 or [^{125}I]PTA-OH was displaced by SQ29548, BM13505 and BM13177 with the same order of potency and similar apparent affinities as in the functional assay, suggesting that these binding sites represent *bona fide* TP receptors.

The vascular endothelium plays a pivotal role in the control of vascular tone and platelet activation [1]. Various endogenous mediators, such as bradykinin and thrombin, stimulate endothelial cells to produce prostacyclin (PGI_2§) and endothelium-derived relaxing factor, both of which are vasodilators and inhibitors of platelet aggregation [1].

Activated platelets release thromboxane A_2 (TXA_2), which is a potent vasoconstrictor and promotes platelet aggregation [2]. Because of the opposing effects of TXA_2 and PGI_2 on platelets and vascular smooth muscle, it has been suggested that an imbalance in the formation of these substances may contribute to the pathophysiology of various disease states such as angina pectoris and atherosclerosis [2, 3].

Preliminary experiments [4, 5] have shown that the TXA_2 mimetic U46619 stimulates PGI_2 release from AG4762 bovine aortic endothelial cells. This release may be important in the feedback control of responses to TXA_2. In this study, we have investigated the antagonist specificity, the radioligand binding characteristics and the effector system of the putative TXA_2 (TP) receptor involved in this response.

MATERIALS AND METHODS

Materials

[5,6-^{3}H]SQ29548 ([^{3}H]SQ29548, 30 Ci/mmol) was obtained from New England Nuclear (Boston, U.K.); 9,11-dimethylmethano-11,12-methano-16(3-[^{125}I]iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15-ω-o-tetranor-TXA_2 ([^{125}I]PTA-OH, 2000 Ci/mmol), [^{3}H]6-keto-prostaglandin (PG)$F_{1\alpha}$ and [^{3}H]-myo-inositol (18 Ci/mmol) were obtained from Amersham International (Amersham, U.K.); 9,11-dideoxy-11,9-epoxymethano-PG $F_{2\alpha}$ (U46619) was from Upjohn Diagnostics (Kalamazoo, MI, U.S.A.). Unlabelled SQ29548 ([1s-[1<a,2<b(5z), 3<b,4<a]] - 7 - [3-[[2-[(phenylamino) carbonyl]-hydrazino]methyl]-7-oxabicyclo-[2.2.1]-hept-2-yl]-5-heptenoic acid) was a kind gift from E.R. Squibb and Sons (Princeton, NY, U.S.A.) and BM13177 (4-[2-(phenylsulphonamido)ethyl]phenoxyacetic acid) and BM13505 (4-[2-(4-chlorobenzene-sulphonamido)ethyl]phenylacetic acid) were gifts from Smith Kline Beecham (Welwyn, U.K.). Rabbit antiserum against 6-keto-$PGF_{1\alpha}$ was a generous gift from Dr

‡ Corresponding author: Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. Tel (021) 414-4511. FAX (021) 414-4509.

§ Abbreviations: BM13505, 4-[2-(4-chlorobenzene-sulphonamido)ethyl]phenylacetic acid; BM13177, 4-[2-(phenylsulphonamido)ethyl]phenoxyacetic acid; [^{125}I]PTA-OH, 9,11-dimethylmethano-16(3-[^{125}I]iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15-ω-o-tetranor-thromboxane A_2; PGI_2, prostacyclin; SQ29548, [1s-[1 < a, 2 < b(5z),3 < b,4 < a]]-7-[3-[[2-[(phenylamino) carbonyl] hydrazino]methyl] - 7 - oxabicyclo - [2.2.1]hept - 2 - yl] - 5-heptenoic acid; TXA_2, thromboxane A_2; TP receptor, TXA_2 receptor; U46619, 9,11-dideoxy-11,9-epoxymethano-prostaglandin $F_{2\alpha}$; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$.

S. Barrows (Department of Clinical Pharmacology, UMDS, London). All other drugs and chemicals were obtained from BDH (Poole, U.K.), Calbiochem (La Jolla, CA, U.S.A.) or the Sigma Chemical Co. (Poole, U.K.).

Cell culture

AG4762 cells were obtained from the cell repository of the National Institute of Aging (U.S.A.). Cells (passage 16–22) were grown to confluency in 80-cm^2 flasks at 37° in a humidified atmosphere containing 7% CO_2 in air. The growth medium was Dulbecco's modified Eagles medium supplemented with 10% foetal calf serum and 1.5 mM glutamine. Cells were subcultured using trypsin (1 mg/mL) and EDTA (0.25 mM) in Dulbecco's phosphate-buffered saline.

Prostacyclin release

Cells were subcultured on to 15-mm multiwells. The multiwell plates were removed from the incubator and maintained at 37° in a water bath for 1 hr prior to the start of the experiment. The medium was removed and replaced with 1 mL Dulbecco's modified Eagle's medium containing drugs as indicated. At the end of the incubation period (10 min unless otherwise stated) this medium was removed for analysis. In antagonist studies, cells were exposed to antagonist for 20 min prior to the addition of agonist. The protein content of each well was determined by a modification of the method of Lowry *et al.* [6] with bovine serum albumin as standard.

Prostacyclin release was measured by radioimmunoassay of its stable hydrolysis product, 6-keto-$PGF_{1\alpha}$, as described by Crossman *et al.* [7]. Preliminary experiments showed that U46619 cross-reacted (*ca.* 25%) with the 6-keto-$PGF_{1\alpha}$ antibody, so a solvent extraction was performed to separate 6-keto-$PGF_{1\alpha}$ from U46619 prior to the radioimmunoassay. SepPak C18 cartridges (Waters Associates) were primed with 2 mL methanol followed by 5 mL distilled water. The sample (150 μL) was applied and the column was washed with 2 mL citrate buffer pH 4.0, 1 mL water and then 12 mL chloroform. The 6-keto-$PGF_{1\alpha}$ was then eluted with 12 mL methanol, evaporated to dryness and resuspended in 150 μL 50 mM Tris–HCl pH 7.4. The recovery of 6-keto-$PGF_{1\alpha}$ was typically 90–95%, whereas >98% of U46619 was lost.

Calcium fluorimetry

Cells were grown on coverslips and loaded with 1 μM Fura-2/AM for 45 min at 37° [8]. Coverslips were washed and placed across the diagonal of a quartz cuvette containing 1.5 mL 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4 in a Spex dual wavelength spectrofluorimeter. Excitation was at 340 nm and 380 nm and emission was monitored at 500 nm. Drugs were added in 1.5–15-μL volumes and mixed using a pasteur pipette. At the end of each experiment the system was calibrated by the addition of 10 μM ionomycin followed by 5 mM $MnCl_2$ and intracellular Ca^{2+} concentrations were calculated according to Grynkiewicz *et al.* [9].

Phosphoinositide hydrolysis

Cells were subcultured in 15-mm multiwells and loaded with [^{3}H]inositol during a 24 hr incubation in Earle's balanced salt solution containing minimal essential amino acids and 5 μCi/mL [^{3}H]*myo*-inositol. Cells were preincubated with 5 mM LiCl for 10 min, and then drug/vehicle was added for a further 10 min incubation. Inositol phosphates were extracted in chloroform:methanol (1:2, v:v) and recovered from Dowex anion exchange columns according to the method of Berridge *et al.* [10].

Radioligand binding

Preparation of AG4762 cell membranes. Confluent cells were harvested mechanically in Dulbecco's phosphate-buffered saline containing 10 μM indomethacin, to prevent formation of endogenous prostanoids. Pelleted cells were stored at −80° prior to membrane preparation. Thawed pellets were homogenized in 50 mM Tris–HCl pH 7.4 containing 0.2 M sucrose by 20 strokes of a tightly fitting Dounce homogenizer and the homogenate was centrifuged at 1500 *g* for 10 min. The supernatant was centrifuged at 30,000 *g* for 15 min and the resulting membrane pellet was washed in 50 mM Tris–HCl pH 7.4, 0.25 mM EDTA, 10 μM indomethacin by three subsequent resuspension and centrifugation steps. The final pellet was resuspended in 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 10 μM indomethacin to a protein concentration of *ca.* 1 mg/mL and frozen in aliquots at −80°.

Binding assays. Incubations in a final volume of 200 μL were set up containing 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 10 μM indomethacin, 2.5 nM [^{3}H]SQ29548 or 0.1 nM [^{125}I]PTA-OH, competing ligands as appropriate, and 50–100 μg membrane protein. Non-specific binding of [^{3}H]SQ29548 was defined using 100 μM BM13177 and non-specific binding of [^{125}I]PTA-OH was defined using 10 μM U46619. Samples were routinely incubated for 30 min at 20°. At the end of this period the samples were filtered on to Whatman GF/B glass fibre filters and washed with 3 × 3.5 mL ice-cold 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ using a Brandel cell harvester.

Data analysis

IC_{50} values for antagonist inhibition curves were obtained by non-linear regression analysis (P. Fit, Fig. P Software Corporation, Durham, NC, U.S.A.); in all cases the data could be adequately described by a simple Langmuir isotherm. Data were compared using Student's *t*-test; a confidence level of 95% was taken to be significant.

RESULTS

Prostacyclin release

The TP receptor agonist, U46619, produced a dose-dependent increase in the release of PGI_2 from AG4762 cells, with an EC_{50} of ≈ 100 nM (log EC_{50} = −7.0 ± 0.8, means ± SEM, N = 6). Maximal concentrations of U46619 (10 μM) or bradykinin (10 nM) produced significant increases in PGI_2 release. The

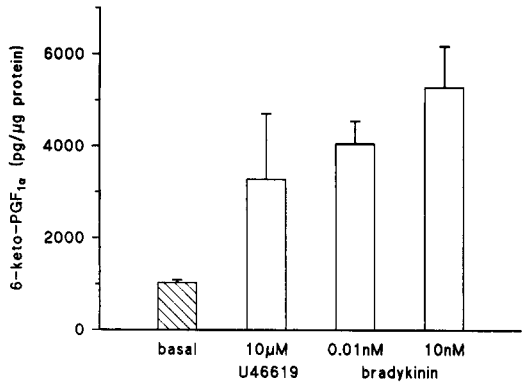

Fig. 1. Prostacyclin release from AG4762 cells. Release of prostacyclin was measured by radioimmunoassay of its stable hydrolysis product, 6-keto-PGF$_{1\alpha}$ (see Materials and Methods). The hatched bar represents basal levels of 6-keto-PGF$_{1\alpha}$ and the open bars represent the increase in 6-keto-PGF$_{1\alpha}$ over basal levels in the presence of 10 μM U46619, 0.01 nM and 10 nM bradykinin. Data are expressed as mean ± SEM from six (0.01 nM bradykinin), eight (10 nM bradykinin) or 10 (basal and U46619) separate experiments.

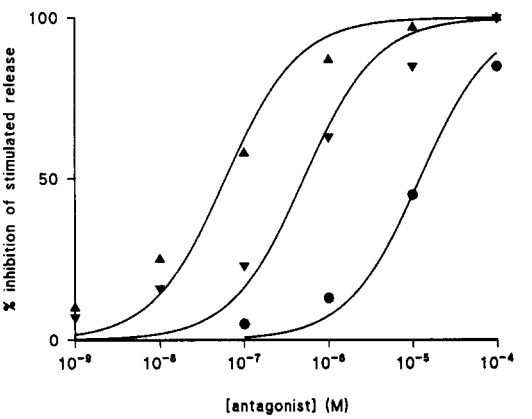

Fig. 2. Inhibition of U46619-stimulated release of PGI_2 from AG4762 cells by SQ29548 (▲), BM13505 (▼) and BM13177 (●). Experiments were carried out as described in Materials and Methods. Each point is the mean of four determinations and the solid lines represent the best fit of a simple Langmuir isotherm to the data; the data are from a single experiment, representative of seven.

maximal response to bradykinin was consistently greater than the maximal response to U46619, which was similar in magnitude to the response to 0.01 nM bradykinin (Fig. 1). Basal release of PGI_2 was 1030 ± 60 pg 6-keto-PGF$_{1\alpha}$/ng protein (means ± SEM, N = 10) under these conditions, and the responses to 10 μM U46619, 10 nM bradykinin and 0.01 nM bradykinin represented 3.2-, 5.1- and 3.9-fold increases over basal, respectively.

The TXA_2 antagonists BM13177, BM13505 and SQ29548 inhibited PGI_2 release stimulated by 10 μM U46619 with the following order of potency: SQ29548 (IC_{50} ≈ 40 nM) > BM13505 (IC_{50} ≈ 740 nM) > BM13177 (IC_{50} ≈ 23 μM) (Fig. 2; Table 1).

Ca^{2+} *mobilization*

The resting level of Ca^{2+} in AG4762 cells was 60 ± 10 nM (mean ± SEM, N = 20). U46619 (10 μM) produced a small Ca^{2+} transient which was produced rapidly and declined rapidly back towards basal Ca^{2+} levels (Fig. 3). Bradykinin also produced an increase in intracellular Ca^{2+} concentration in these cells. The peak response to 10 nM bradykinin was consistently followed by a small but prolonged "plateau phase" of elevated Ca^{2+}. A similar plateau was occasionally observed in response to 0.01 nM bradykinin, but this was not a consistent feature of the response.

The peak response to 10 μM U46619 corresponded to an increase in the intracellular Ca^{2+} concentration of 158 ± 21 nM (mean ± SEM, N = 10). This was again very similar to the peak response obtained with 0.01 nM bradykinin, which produced an increase in the intracellular Ca^{2+} concentration of 203 ± 15 nM (mean ± SEM, N = 6). The increase in intracellular Ca^{2+} concentration produced by 10 nM bradykinin was 775 ± 40 nM (mean ± SEM, N = 10).

Phosphoinositide hydrolysis

The phosphoinositide responses that could be measured in these cells were very small. The basal level of [^{3}H]inositol phosphates was 412 ± 33 dpm/μg protein (mean ± SEM, N = 4). In the presence of 10 nM bradykinin this level was significantly increased by 43% to 588 ± 81 dpm/μg protein (mean ± SEM, N = 4). However, 10 μM U46619 and 0.01 nM bradykinin did not produce any significant increase in phosphoinositide hydrolysis, the levels of [^{3}H]inositol phosphates being 500 ± 198 dpm/μg protein (mean ± SEM, N = 4) bradykinin was 775 ± 40 nM (mean ± SEM, N = 10).

Radioligand binding

Under the conditions used in these experiments the total binding of [^{125}I]PTA-OH was typically 2000 dpm/sample, of which 35% represented "specific" binding. Total binding of [^{3}H]SQ29548 was typically 300 dpm/sample, of which 45% represented "specific" binding.

Both [^{125}I]PTA-OH and [^{3}H]SQ29548 appeared to bind to homogeneous populations of sites and self-competition curves were well described by a single site model of binding. Analysis of these self-competition curves [11] yielded the following estimates of affinity and binding capacity: for [^{125}I]-PTA-OH, log $K = -9.50 \pm 0.18$ (mean ± SEM, N = 4) and $B_{max} = 24 \pm 2.4$ fmol/mg protein (mean ± SEM, N = 4); for [^{3}H]SQ29548, log $K = -8.00 \pm 0.08$ (mean ± SEM, N = 4) and $B_{max} = 26 \pm 3.4$ fmol/mg protein (means ± SEM, N = 4).

The ability of the three TXA_2 antagonists, SQ29548, BM13505 and BM13177, to inhibit the specific binding of these radioligands was investigated. In each case the antagonist displacement curve was consistent with binding to a single site.

Table 1. Estimates of log IC_{50} values for three TXA_2 antagonists in AG4762 cells obtained from functional and binding assays

Assay system	log IC_{50} (M)		
	SQ29548	BM13505	BM13177
Inhibition of PGI_2 release	−7.40 ± 0.48, N = 8	−6.13 ± 0.02, N = 8	−4.70 ± 0.13, N = 7
Inhibition of [^{3}H]SQ29548 binding	−8.50 ± 0.27, N = 5	−6.70 ± 0.12, N = 4	−4.90 ± 0.43, N = 4
Inhibition of [^{125}I]PTA-OH binding			
No preincubation	−5.68 ± 0.13, N = 6	−7.50 ± 0.14, N = 6	−3.90 ± 0.13, N = 4
Preincubation	−7.90 ± 0.70, N = 5	−6.80 ± 0.13, N = 4	−5.90 ± 0.10, N = 4

Assays were carried out as described in Materials and Methods. In all cases, samples were incubated for 30 min at 20° following the addition of radioligand. In [^{3}H]SQ29548 assays radioligand and unlabelled ligand were added simultaneously. In [^{125}I]PTA-OH binding studies, radioligand and unlabelled ligand were either added simultaneously ("no preincubation") or membranes were preincubated with the unlabelled antagonists for 60 min before the addition of the radioligand ("preincubation").

Data are expressed as means ± SEM from N separate experiments.

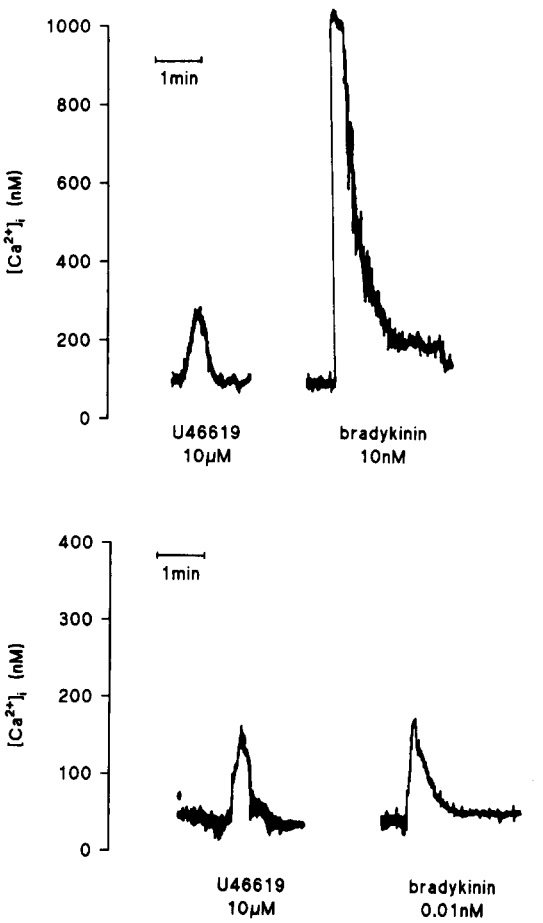

Fig. 3. Effect of U46619 and bradykinin on intracellular calcium concentration in AG4762 cells. Intracellular calcium concentration was measured as described in Materials and Methods. These traces are from single experiments, representative of 10.

However, in experiments where radioligands and competing ligands were added to the membranes simultaneously, different orders of potency were obtained against [^{125}I]PTA-OH or [^{3}H]SQ29548. Using [^{125}I]PTA-OH, the apparent order of potency was BM13505 ($IC_{50} \approx 32$ nM) > SQ29548 ($IC_{50} \approx 2.1$ μM) > BM13177 ($IC_{50} \approx 130$ μM) (Table 1). Using [^{3}H]SQ29548, the order of potency obtained was SQ29548 ($IC_{50} \approx 32$ nM) > BM13505 ($IC_{50} \approx 200$ nM) > BM13177 ($IC_{50} \approx 13$ μM) (Fig. 4; Table 1).

This difference in the apparent orders of potency of the antagonists seems to have occurred because equilibrium had not been achieved in the experiments with [^{125}I]PTA-OH. When AG4762 cell membranes were preincubated with the unlabelled antagonists for 60 min before the addition of [^{125}I]PTA-OH (with incubation for a further 30 min), the following order of potency was obtained: SQ29548 ($IC_{50} \approx 13$ nM) > BM13505 ($IC_{50} \approx 160$ nM) > BM13177 ($IC_{50} \approx 1.3$ μM) (Table 1).

The agonist U46619 inhibited the specific binding of [^{3}H]SQ29548 with an IC_{50} of ≈40 nM (log IC_{50} = −7.4 ± 0.15, mean ± SEM, N = 5).

DISCUSSION

These results clearly demonstrate that the TXA_2 mimetic U46619 stimulates PGI_2 release from AG4762 bovine aortic endothelial cells via an interaction with TP receptors. This release presumably represents a feedback mechanism whereby the vasoconstrictor and pro-aggregatory effects of TXA_2 may be reduced by the potent vasodilator and anti-aggregatory effects of PGI_2. In this regard, it is interesting that in disease states where there is an increased level of TXA_2, PGI_2 levels are also raised [12]. An increase in PGI_2 synthesis has been thought to be due to the "redirection" of the cyclic endoperoxide intermediate PGH_2, which is produced in platelets but subsequently converted to PGI_2 by endothelial cells [12]. However, endothelial cells are clearly capable of synthesizing PGI_2 from endogenous arachidonic acid, and this synthesis can be stimulated

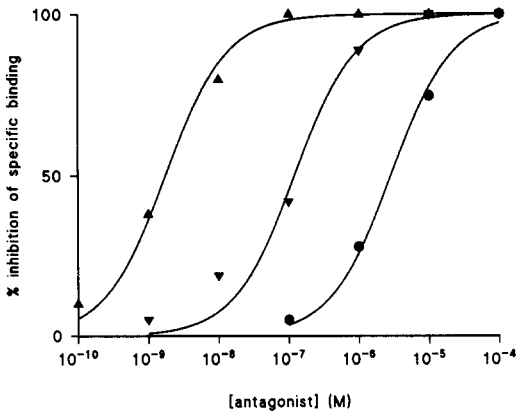

Fig. 4. Inhibition of the specific binding of [^{3}H]SQ29548 to AG4762 cell membranes by SQ29548 (▲), BM13505 (▼) and BM13177 (●). Experiments were carried out as described in Materials and Methods. Each point is the mean of two determinations and the solid lines represent the best fit of a simple Langmuir isotherm to the data; the data are from a single experiment, representative of four.

by TXA_2. It seems very likely that this mechanism contributes to the increase in PGI_2 which accompanies an increase in TXA_2.

In contrast to the present findings, Sung *et al.* [13] have reported that U46619 *inhibits* release of PGI_2 from endothelial cells obtained from bovine pulmonary artery. However, human umbilical arteries release PGI_2 when stimulated with U46619 [14], suggesting that umbilical artery endothelial cells behave in the same way as AG4762 cells. The reason for the apparent difference is unclear, but it is possible that endothelial cells display a regional heterogeneity in their response to TP receptor stimulation.

The order of antagonist potency obtained for the inhibition of U46619-stimulated release of PGI_2 from endothelial cells was SQ28548 > BM13505 > BM13177. The same order of potency has been obtained in functional studies on human and rabbit platelets, rabbit aorta and guinea-pig bronchi [15]. The existence of TP receptor subtypes has been much debated [3, 15–18] but it is now clear that the primary structure of TP receptors from platelets and vascular smooth muscle is the same [19]. With the antagonists used here, the endothelial cell receptors are indistinguishable from those in other tissues.

The TP receptors on AG4762 cells also seem to be similar to other TP receptors with regard to their second messenger system. In platelets and vascular smooth muscle cells, TP receptors increase intracellular Ca^{2+} levels via activation of phospholipase C, hydrolysis of phosphatidylinositol-4,5-bisphosphate and liberation of inositol-1,4,5-triphosphate [3, 17]. This second messenger system is also utilized by those agents which stimulate PGI_2 synthesis in endothelial cells, such as thrombin and bradykinin [1, 8, 20, 21], suggesting that these agents may well stimulate phospholipase A_2 via an increase in intracellular Ca^{2+}. U46619 produces an increase in intracellular Ca^{2+} in AG4762 cells but it does not produce any significant stimulation of phosphoinositide hydrolysis. However, a concentration of bradykinin which produces the same stimulation of PGI_2 release and the same Ca^{2+} response as the maximal responses to U46619 (0.01 nM bradykinin) is also unable to produce any significant increase in levels of inositol phosphates. These data are consistent with the hypothesis that both U46619 and bradykinin stimulate PGI_2 release by mobilization of Ca^{2+} via stimulation of phosphoinositide hydrolysis, provided that a measurable Ca^{2+} response can be achieved when the phosphoinositide response is so small as to be indistinguishable from basal. However, other possibilities, such as opening of receptor-operated Ca^{2+} channels or a direct activation of phospholipase A_2, cannot be ruled out.

When ligand binding experiments were carried out under conditions which attempted to ensure that equilibrium had been closely approached, the properties of the [^{3}H]SQ29548 and [^{125}I]PTA-OH binding sites on AG4762 cell membranes appeared very similar. The apparent affinities of TXA_2 antagonists for these sites were also very similar to their apparent affinities in functional assays, suggesting that these binding sites do indeed represent *bona fide* TP receptors. Furthermore, the EC_{50} for U46619 stimulating PGI_2 release was not significantly different from its IC_{50} for inhibiting the specific binding of [^{3}H]SQ29548.

Previous attempts to determine the binding characteristics of endothelial cell TP receptors have yielded contradictory results, with SQ29548 having a high affinity for sites labelled with [^{3}H]SQ29548 [22] but a much lower affinity for sites labelled with [^{125}I]PTA-OH [13]. The same discrepancy was apparent in the present study; in experiments where radioligand and competing ligand were added simultaneously, different orders of antagonist potency were obtained using the different radioligands and SQ29548 appeared to have a much higher affinity in experiments with [^{3}H]SQ29548 than in experiments with [^{125}I]PTA-OH. However, [^{3}H]SQ29548 and [^{125}I]PTA-OH appeared to label the same number of sites and the populations of sites did not appear to be heterogeneous.

The anomalies in the competition experiments with [^{125}I]PTA-OH were consistent with those predicted for competition experiments where equilibrium has not been achieved and in which the radioligand binds faster than the competing ligand [23]. This hypothesis was tested by preincubating AG4762 cell membranes with unlabelled ligands prior to the addition of [^{125}I]PTA-OH and under these conditions the antagonist order of potency was indeed the same as that obtained using [^{3}H]SQ29548 or for the inhibition of U46619-stimulated PGI_2 release. However, it was not established conclusively that true equilibrium conditions had been achieved in any of the assays, and for this reason affinities have not been calculated from the IC_{50} values.

Equilibration of [^{125}I]PTA-OH and competing ligands does not appear to be a problem in binding assays performed in platelets [15]. This suggests that the binding kinetics of these ligands in platelets and endothelial cells must be rather different and

preliminary experiments indicate that while the off-rates of [^{125}I]PTA-OH and [^{3}H]SQ29548 in platelet membranes are very similar, in AG4762 cell membranes the off-rate of [^{125}I]PTA-OH is much faster than that of [^{3}H]SQ29548 (Hunt and Keen, unpublished). Differences in the kinetics of TXA_2 antagonist binding between rat platelets and smooth muscle cells have also been reported [24].

It is not only in binding assays that these differences in kinetics may become significant. The order of antagonist potency for the inhibition of U46619-stimulated PGI_2 release from AG4762 cells in a continuous flow column perfusion assay was found to be BM13505 > SQ29548 > BM13177: the same as that found for inhibition of [^{125}I]PTA-OH binding under non-equilibrium conditions [5]. Furthermore, the selectivity of the novel TP receptor antagonist GR32191 appears to be due to the fact that it behaves as an irreversible antagonist at receptors on platelets, but as a reversible antagonist at receptors on vascular smooth muscle [17]. It may be that kinetic differences underly some of the reports of TP receptor heterogeneity.

This study has demonstrated the existence of TP receptors on endothelial cells using both functional and radioligand binding assays. These receptors mediate release of PGI_2 and are likely to be of importance in regulating the activity of TXA_2 *in vivo*. The endothelial cell TP receptors seem to be very similar to TP receptors which have been characterized in platelets and smooth muscle cells, although they may exhibit some small differences in the apparent binding kinetics of various ligands.

REFERENCES

1. Gryglewski RJ, Botting RM and Vane JR, Mediators produced by the endothelial cell. *Hypertension* **12**: 530–548, 1988.
2. Bunting S, Moncada S and Vane JR, The prostacyclin–thromboxane A_2 balance: pathophysiological and therapeutic implications. *Br Med Bull* **39**: 271–276, 1983.
3. Halushka PV, Mais DE, Mayeux PR and Morinelli TA, Thromboxane, prostacyclin and leukotriene receptors. *Annu Rev Pharmacol Toxicol* **29**: 213–239, 1989.
4. Hunt JA, Hallam TJ and MacDermot J, Thromboxane triggers release of prostacyclin from cultured aortic endothelial cells. *Br J Pharmacol* **96**: 170P, 1989.
5. Hunt JA, MacDermot J and Keen M, Characterization of a thromboxane receptor mediating prostacyclin release from cultured bovine aortic endothelial cells. *Eur J Pharmacol* **183**: 2142–2143, 1990.
6. Lowry OH, Rosebrough NJ, Farr AL and Randall PJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
7. Crossman DC, McEwan J, MacDermot J, MacIntyre I and Dollery CT, Human calcitonin gene-related peptide activates adenylate cyclase and releases prostacyclin from human umbilical vein endothelial cells. *Br J Pharmacol* **92**: 695–703, 1987.
8. Hallam TJ, Pearson JD and Needham LA, Thrombin stimulated elevation of human endothelial cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochem J* **251**: 243–249, 1988.
9. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
10. Berridge MJ, Downes P and Hanley MR, Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J* **206**: 587–595, 1982.
11. DeBlasi A, O'Reilly K and Motulsky HJ, Calculating receptor number from binding experiments using the same compound as radioligand and competitor. *Trends Pharmacol Sci* **10**: 227–229, 1989.
12. Gresele P, Deckmyn H, Nenci GG and Vermylen J, Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. *Trends Pharmacol Sci* **12**: 158–163, 1991.
13. Sung C-P, Arleth AJ and Berkowitz BA, Endothelial thromboxane receptors: biochemical characterization and functional implications. *Biochem Biophys Res Commun* **158**: 326–333, 1989.
14. Bjoro K, Prostacyclin and thromboxane formation in human umbilical arteries following stimulation with vasoactive substances. *Prostaglandins* **31**: 699–714, 1986.
15. Swayne GTG, Maguire J, Dolan J, Raval P, Dane G, Greener M and Owen DAA, Evidence for homogeneity of thromboxane A_2 receptors using structurally different antagonists. *Eur J Pharmacol* **152**: 311–319, 1988.
16. Mais DE, Saussy DL, Chaikhouni A, Kochel PJ, Knapp DR, Hamanaka N and Halushka PV, Pharmacological characterization of human and canine thromboxane A_2/prostaglandin H_2 receptors in platelets and blood vessels: evidence for different receptors. *J Pharmacol Exp Ther* **233**: 418–424, 1985.
17. Giles H, More selective ligands at eicosanoid receptor subtypes improve prospects in inflammatory and cardiovascular research. *Trends Pharmacol Sci* **11**: 301–304, 1990.
18. Tymkewycz PM, Jones RL, Wilson NH and Marr CG, Heterogeneity of thromboxane A_2 (TP-) receptors: evidence from antagonist but not agonist potency measurements. *Br J Pharmacol* **102**: 607–614, 1991.
19. Hirata M, Hayashi Y, Ushikubi F, Yokata Y, Kageyama R, Nakanishi S and Narumiya S, Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature* **349**: 617–620, 1991.
20. Pollock WK, Wreggett KA and Irvine RF, Inositol phosphate production and Ca^{2+} mobilization in human umbilical vein endothelial cells stimulated by thrombin and histamine. *Biochem J* **256**: 371–376, 1988.
21. Bartha K, Muller-Peddinghaus R and Van Rooijen LAA, Bradykinin and thrombin effects on polyphophoinositide hydrolysis and prostacyclin production in endothelial cells. *Biochem J* **263**: 149–155, 1989.
22. Hanasaki K, Nakano K, Kasai H, Kurihara H and Arita H, Identification of thromboxane A_2 receptors in cultured vascular endothelial cells of rat aorta. *Biochem Biophys Res Commun* **151**: 1352–1357, 1988.
23. Motulsky HJ and Mahan LC, The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol* **25**: 1–9, 1984.
24. Hanasaki K, Nakano K, Kasai H and Arita H, Biochemical characterization and comparison of rat thromboxane A_2/prostaglandin H_2 receptors in platelets and cultured aortic smooth muscle cells. *Biochem Pharmacol* **38**: 2967–2976, 1989.