Biochemical Pharmacology, Vol. 59, pp. 1521–1530, 2000.
© 2000 Elsevier Science Inc. All rights reserved.


ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00296-3

# Shifts in the Affinity Distribution of One Class of Seven-Transmembrane Receptors by Activation of a Separate Class of Seven-Transmembrane Receptors

*Yasmine Djellas,* Kostas Antonakis† and Guy C. Le Breton*‡*

*Department of Pharmacology, University of Illinois at Chicago, Chicago, IL 60612, U.S.A.; and †Ecole Nationale Superieure de Chimie de Paris, Laboratoire de Vectorologie Moleculaire, Paris 75231, France

**ABSTRACT.** We have demonstrated previously that activation of thrombin receptors causes increased $G\alpha_q$ coupling to thromboxane $A_2$ receptors and increased thromboxane $A_2$ receptor ligand affinity. These results led to the hypothesis that thrombin receptor activation stimulates $G\alpha_q$ redistribution to thromboxane $A_2$ receptors, thereby shifting them to a higher affinity state. The present study investigated three questions regarding this inter-receptor signaling phenomenon: (i) does activation of thrombin receptors cause a redistribution of thromboxane $A_2$ receptor subpopulations; (ii) does inter-receptor signaling require that participating receptors couple to the same family of G-protein α-subunits; and (iii) does inter-receptor signaling occur in cell types other than platelets? It was found that thrombin receptor activation caused a shift in the thromboxane $A_2$ receptor binding data from a one-site model to a two-site model ($K_i$ = 0.5 μM vs $K_i$ = 10 nM and 1.1 μM for the antagonist 4-[2-[[(4-chlorophenyl)sulfonyl]amino]ethyl]benzeneacetic acid (BM13.505) and $K_i$ = 2.5 μM vs $K_i$ = 29.5 nM and 2.6 μM for the agonist 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619). It also was found that activation of prostaglandin $D_2$ receptors also caused a shift of prostacyclin receptor binding data from a one-site model ($IC_{50}$ = 10.1 nM) to a two-site model ($IC_{50}$ = 3.3 and 12.5 nM). The physiological manifestation of this inter-receptor signaling between prostacyclin and prostaglandin $D_2$ receptors was a synergistic inhibition of human platelet aggregation. Finally, the present results established that activation of endothelial cell thrombin receptors shifts thromboxane $A_2$ receptor affinity from $K_i$ = 0.8 μM (control) to $K_i$ = 0.2 μM (thrombin receptor-activating peptide), indicating that cells other than platelets have the capability to signal between seven-transmembrane receptors. BIOCHEM PHARMACOL **59**;12:1521–1530, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** $TXA_2$ receptors; G-proteins; inter-receptor signaling; synergism

Cells process information from their environment through activation of different membrane receptors. One major group of receptors constitutes the family of seven-transmembrane G-protein§-coupled receptors. Stimulation of such receptors by an agonist will lead to activation of their associated G-protein(s) and propagation of the signal through heterotrimer dissociation into a Gα subunit and a Gβγ dimer. Each of these subunits can then activate specific effector sites [1–4]. Whereas different receptors seem to function through distinct signal transduction pathways, it is also apparent that activation of a single pathway can modulate one or more other signaling cascades. For example, activation of adenosine $A_1$ receptors in guinea-pig cerebral cortex potentiates inositol phosphate accumulation induced by histamine $H_1$ receptors through phospholipase C activation by Gβγ released from $G\alpha_i$ subunits [5, 6]. Other results have demonstrated that m2- and m4-muscarinic, $\alpha_2$-adrenergic, or $D_2$-dopaminergic receptor activation increases ATP-induced arachidonic acid release in a protein kinase C-dependent mechanism [7].

In addition to these downstream "cross-talk" events, we recently identified a novel mechanism by which seven-transmembrane receptors can communicate directly via a redistribution of their G-protein(s) [8]. Specifically, it was found that activation of platelet thrombin receptors [9–12] causes an increase in $G\alpha_q$ association to $TXA_2$ receptors [13–15] and an increase in average $TXA_2$ receptor ligand affinity. However, this previously observed increase in average affinity could have derived from either an increase in affinity of all $TXA_2$ receptors or a redistribution in affinities within pre-existing $TXA_2$ receptor subpopulations. In the present study, competition-binding experi-

‡ Corresponding author: Guy C. Le Breton, Ph.D., Department of Pharmacology, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Ave. (M/C 868), Chicago, IL 60612. Tel. (312) 996-4929; FAX (312) 996-1225; E-mail: gcl@tigger.uic.edu

§ *Abbreviations*: G-protein, heterotrimeric guanine nucleotide binding protein; $TXA_2$, thromboxane $A_2$; $PGI_2$, prostacyclin; $PGD_2$, prostaglandin $D_2$; TRAP, thrombin-receptor activating peptide; PAF, platelet-activating factor; FBS, fetal bovine serum; HPAEC, human pulmonary artery endothelial cells; and CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonic acid.

Received 12 August 1999; accepted 12 November 1999.

ments were performed to further investigate these separate possibilities. Our results demonstrated that TRAP [9–12, 16–18] causes the appearance of new high-affinity binding sites, providing evidence that there is indeed a redistribution of affinities within the $TXA_2$ receptor pool.

Next, experiments were performed to investigate whether coupling to a common G-protein family is a requirement for the ability of one activated receptor to increase the affinity of a separate receptor. This study evaluated two separate receptor pairs, i.e. those that are $G_s$-coupled ($PGD_2$ [19, 20]:$PGI_2$ [21–24] receptors) and those that are $G_q$-coupled (thrombin:$TXA_2$ [25–28] receptors). It was found that inter-receptor signaling occurs only between receptors that share a common G-protein, i.e. between $PGD_2$ and $PGI_2$ receptors or between thrombin and $TXA_2$ receptors. As was observed for the $TXA_2$: thrombin receptor pair [8], inter-receptor signaling between $PGD_2$ and $PGI_2$ receptors was associated with a synergistic effect on platelet aggregation.

Finally, experiments were conducted to determine whether inter-receptor signaling is limited only to platelets or whether it extends to other cell types as well. To address this issue, signaling between thrombin and $TXA_2$ receptors was measured in HPAEC [29–32]. The results from these studies demonstrated that activation of thrombin receptors leads to increases in $TXA_2$ receptor ligand affinity. These findings, therefore, indicated that endothelial cells also have the capacity for signaling between separate receptor pathways.

## MATERIALS AND METHODS

### *Materials*

Outdated platelet concentrates were obtained from Heartland Blood Services. HPAEC, modified MCDB 131 medium, and trypsin 0.025%/EDTA 0.01% were purchased from the Clonetics Corp. [$^3$H][1S-[1x, 2B, 4x]]-7-[3-[ [2-[(phenylamino]-carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.2]-hept-2-yl]-5-heptenoic acid ([$^3$H]SQ29, 548) was purchased from DuPont/NEN. 9,11-Dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619) and SQ29,548 were obtained from Cayman Chemicals. [$^3$H]Iloprost and iloprost were purchased from Amersham Life Science. 4- [2-[[(4-Chlorophenyl]sulfonyl]amino]ethyl]benzeneacetic acid (BM13.505) was supplied by Dr. K. Stegmeier, Boehringer Mannheim GmbH. TRAP refers to the first 6 amino acids of the new amino terminus revealed after thrombin cleavage, i.e. SFLLRN (TRAP [42–47]; this peptide was purchased from Research Genetics. CHAPS, EDTA, FBS, $PGI_2$, $PGD_2$, and indomethacin were purchased from the Sigma Chemical Co.

### *[$^3$H]SQ29,548 Binding to Solubilized Platelet Membranes*

Platelet membranes were prepared from outdated platelet concentrates and solubilized in 10 mM CHAPS as previously described [15]. The CHAPS concentration was adjusted to 2 mM, and [$^3$H]SQ29,548 binding was performed by using a filtration-binding assay procedure [15, 33, 34]. Nonspecific binding was determined by using a 1000-fold molar excess of the unlabeled SQ29,548 (2 μM).

### *Competition of [$^3$H]SQ29,548 Binding to Solubilized Platelet Membranes*

Solubilized platelet membranes were treated with vehicle or 50 μM TRAP for 3 min and then incubated until equilibrium was reached (20 min) with 2 nM [$^3$H]SQ29,548 in the presence of various concentrations of BM13.505 (1 nM to 50 μM) [35] or U46619 (0.1 nM to 100 μM) [36]. Nonspecific binding was determined using a 1000-fold molar excess of unlabeled SQ29,548 (2 μM) and was typically 5–10% of the total binding. The data were analyzed for one- and two-site fits using the program PRIZM (Graphpad). The two-site model was accepted only if it was statistically a significantly ($P < 0.05$) better fit than the single site. The following parameters were derived: the $IC_{50}$ (concentration of ligand that competes for 50% of the specifically bound [$^3$H]SQ29,548) and the Hill coefficients. The $K_i$ values for BM13.505 and U46619 were calculated from their $IC_{50}$ values by the method of Cheng and Prusoff [37].

### *[$^3$H]Iloprost Binding to Platelet Membranes*

Platelet membranes were prepared as described [15] from outdated platelet concentrates. Binding of the $PGI_2$ receptor agonist [$^3$H]iloprost [22, 23] was assessed by using a filtration-binding assay procedure [15]. Nonspecific binding was determined by using a 10,000-fold molar excess of the unlabeled iloprost (10 μM), and specific binding was typically 66 ± 3% (N = 16) of total binding.

### *Competition of [$^3$H]Iloprost Binding to Platelet Membranes*

Platelet membranes were treated with vehicle or 10 nM $PGD_2$ for 3 min and then incubated until equilibrium was reached (30 min) with 1 nM [$^3$H]iloprost in the presence of various concentrations of iloprost (0.01 nM to 1 μM) [22, 23]. Nonspecific binding was determined using a 10,000-fold molar excess of unlabeled iloprost (10 μM) and was typically 25–30% of the total binding. The data were analyzed for one- and two-site fits using the program PRIZM (Graphpad). The two-site model was accepted only if it was statistically a significantly ($P < 0.05$) better fit than the single site. The following parameters were derived: the $IC_{50}$ (concentration of ligand that competes for 50% of the specifically bound [$^3$H]iloprost) and the Hill coefficients. Since the binding experiments were performed at a ligand concentration well below the published $K_d$ of iloprost [22, 23], the $K_i$ value for iloprost can be estimated to be equal to its $IC_{50}$.

### *Platelet Aggregation*

Platelet-rich plasma was prepared from citrate–phosphate–dextrose-anticoagulated human blood as described [38]. The platelet-rich plasma was incubated for 3 min with 10 μM indomethacin to prevent endogenous $TXA_2$ production. Aggregation was stimulated with TRAP at a concentration that yielded approximately 80% of the maximal aggregation response (submaximal dose), and the aggregation response was measured by the turbidimetric method [39] using a model 400 Lumi-aggregometer (Chronolog Corp.). $PGI_2$ and $PGD_2$ (1–3 nM) were added 1 min prior to the addition of TRAP.

### *[$^3$H]SQ29,548 Binding to Intact HPAEC*

HPAEC, used between passages four and six, were grown in modified MCDB 131 medium supplemented with 10% FBS in 12-well tissue culture plates. The wells were washed twice with PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) and treated with vehicle or 50 μM TRAP for 3 min. The wells were washed, and the binding reagents were added: 2 nM [$^3$H]SQ29,548 plus vehicle (total binding) or plus 2 μM SQ29,548 (nonspecific binding). After 20 min of equilibration at room temperature, the plates were washed once with PBS to remove unbound ligand, and the cells were harvested using trypsin/EDTA. The suspended cells were then filtered on Whatman GF/C filters, washed twice with ice-cold PBS, and counted for [$^3$H]SQ29,548. The counts were normalized for cell number in each well. Typically, the percentage of specific binding was 55 ± 7% (N = 8).

### *Competition of [$^3$H]SQ29,548 Binding to Intact HPAEC*

HPAEC, used between passages four and six, were grown in modified MCDB 131 medium supplemented with 10% FBS in tissue culture flasks to 80–90% confluence. The cells were harvested using 2.5 mM EDTA in PBS, washed to remove residual EDTA, and resuspended in PBS containing 5 mM $MgCl_2$ to a final concentration of $10^6$ cells/mL. The cell suspension (100 μL) was treated with vehicle or 50 μM TRAP for 3 min, and the binding reagents were added: 2 nM [$^3$H]SQ29,548 plus vehicle (total binding), or plus various concentrations of U46619 (1 nM to 10 μM). After 20 min of equilibration at room temperature, the cells were filtered on Whatman GF/C filters, washed twice with ice-cold PBS, and counted for [$^3$H]SQ29,548. Nonspecific binding was determined using a 1000-fold molar excess of unlabeled SQ29,548 (2 μM), and the percentage of specific binding was 30 ± 3% (N = 7). The data were analyzed for one- and two-site fits using the program PRIZM (Graphpad). The two-site model was accepted only if it was statistically a significantly ($P < 0.05$) better fit than the single site. The following parameters were derived: the $IC_{50}$ (concentration of ligand that competes for 50% of the specifically bound [$^3$H]SQ29,548) and the Hill coefficients. The $K_i$ value for U46619 was calculated from its $IC_{50}$ value by the method of Cheng and Prusoff [37] using 7 nM as $K_d$ for SQ29,548 [15].

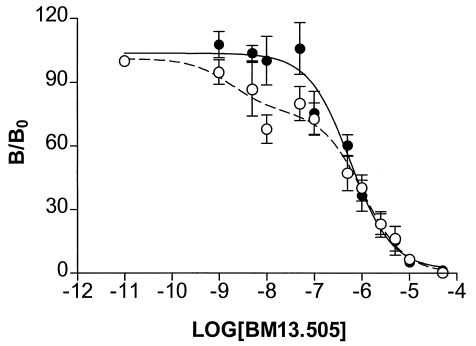


**FIG. 1. Effect of TRAP on the competition of [$^3$H]SQ29,548 binding to solubilized platelet membranes by BM13.505. Solubilized platelet membranes were incubated with vehicle (●) or 50 μM TRAP (○) for 3 min, and [$^3$H]SQ29,548 binding was measured in the presence of increasing concentrations of BM13.505, as described in Materials and Methods. The percentage of specific binding is plotted as a function of the added BM13.505 concentration. Each point represents the mean ± SEM of three separate experiments done in triplicate (when error bars do not appear, they are smaller than the symbol). B = [$^3$H]SQ29,548 specific binding at a given BM13.505 concentration. $B_0$ = [$^3$H]SQ29,548 specific binding in the absence of BM13.505.**

### *Statistical Analysis*

Data were analyzed according to Student's paired *t*-test ($P < 0.05$).

## RESULTS

### *Redistribution of $TXA_2$ Receptor Affinities upon Thrombin Receptor Activation*

To elucidate further the effects of thrombin receptor activation on $TXA_2$ receptor affinity, we first performed SQ29,548 [33, 34] competition-binding studies using the $TXA_2$ receptor antagonist BM13.505 [35] as a competing agent. In these experiments, solubilized platelet membranes, which were pretreated with either vehicle or 50 μM TRAP [9–12, 16–18], were incubated with [$^3$H]SQ29,548 and increasing concentrations of BM13.505. It can be seen in Fig. 1 that BM13.505 effectively competed for SQ29,548 binding in both vehicle- and TRAP-treated solubilized platelet membranes. In the absence of TRAP, nonlinear regression analysis of the binding data resolved only a single binding site with an $IC_{50}$ of 0.7 ± 0.2 μM ($K_i$ = 0.5 ± 0.1 μM) and a Hill coefficient of −1.00 ± 0.01 (N = 3). In contrast, TRAP treatment shifted the Hill coefficient to −0.48 ± 0.13 (N = 3), indicating the presence of more than one binding site. On this basis, these latter binding data best fit a two-site model ($P < 0.05$), which described one binding site with an $IC_{50}$ of 13.4 ± 6.6 nM ($K_i$ =

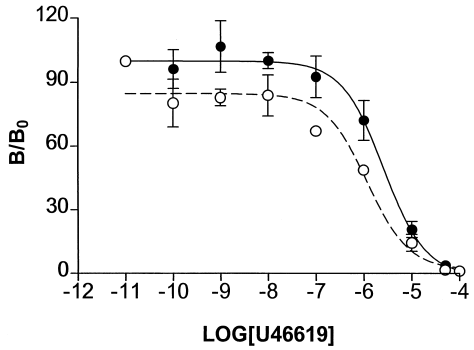


**FIG. 2. Effect of TRAP on the competition of [$^3$H]SQ29,548 binding to solubilized platelet membranes by U46619. Solubilized platelet membranes were incubated with vehicle (●) or 50 μM TRAP (○) for 3 min, and [$^3$H]SQ29,548 binding was measured in the presence of increasing concentrations of U46619, as described in Materials and Methods. The percentage of specific binding is plotted as a function of added U46619 concentration. Each point represents the mean ± SEM of three separate experiments done in triplicate (when error bars do not appear, they are smaller than the symbol). B = [$^3$H]SQ29,548 specific binding at a given U46619 concentration. $B_0$ = [$^3$H]SQ29,548 specific binding in the absence of U46619.**

10.0 ± 4.9 nM) and the second binding site with an $IC_{50}$ of 1.4 ± 0.4 μM ($K_i$ = 1.1 ± 0.3 μM). The appearance of new high-affinity antagonist binding sites after TRAP treatment is consistent with our previous observation describing an increase in average $TXA_2$ receptor affinity upon thrombin receptor activation [8].

We next evaluated whether TRAP induced a similar redistribution in $TXA_2$ receptor affinities for the agonist U46619 [36]. In these experiments, SQ29,548 competition-binding studies were performed using U46619 as a competing agent. Solubilized platelet membranes, which were pretreated with either vehicle or 50 μM TRAP, were incubated with [$^3$H]SQ29,548 and increasing concentrations of U46619. It can be seen in Fig. 2 that U46619 also competed for SQ29,548 binding in both vehicle- and TRAP-treated preparations. In the absence of TRAP, the binding data best fit a one-site model with a Hill coefficient of −0.90 ± 0.25 (N = 3) and an $IC_{50}$ of 3.2 ± 0.9 μM ($K_i$ = 2.5 ± 0.7 μM). On the other hand, it can also be seen (Fig. 2) that TRAP treatment shifted the competition-binding curve to the left, indicating that thrombin receptor activation caused an increase in $TXA_2$ receptor affinity for the agonist U46619. This increased affinity was evidenced by a shift in the $IC_{50}$ from 3.2 μM (vehicle) to 1.5 ± 0.4 μM (TRAP). Furthermore, analysis of the binding data also revealed that TRAP caused a shift in the Hill coefficient from −0.90 (one site) to −0.48 ± 0.13 (N = 3) (multi-site). On this basis, these latter binding data fit a two-site model, which described one binding site with an $IC_{50}$ of 37.9 nM ($K_i$ = 29.5 nM) and the second binding site with an $IC_{50}$ of 3.4 μM ($K_i$ = 2.6 μM). Therefore, with both BM13.505 and U46619, TRAP produced a redistribution of receptor affinities within the $TXA_2$ receptor pool such that new higher affinity sites were generated.

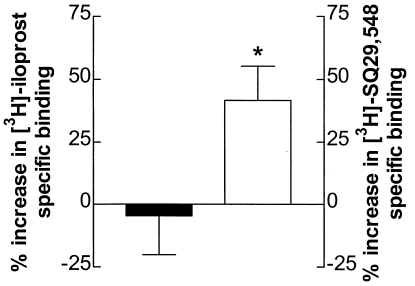


**FIG. 3. Effect of TRAP on [$^3$H]iloprost binding to platelet membranes and [$^3$H]SQ29,548 binding to solubilized platelet membranes. Platelet membranes and solubilized platelet membranes were pretreated with 50 μM TRAP for 3 min. Binding was performed as described under Materials and Methods, and the results are expressed as the percentage variation in [$^3$H]iloprost (solid bar) or [$^3$H]SQ29,548 (open bar) specific binding relative to unstimulated platelet membranes. Experiments were done at least four times in triplicate, and the values represent means ± SEM of all results. The average [$^3$H]iloprost and [$^3$H]SQ29,548 specific binding values in the absence of agonist pretreatment were 65 ± 7 and 298 ± 40 fmol/mg protein, respectively. Statistical analyses measuring the effect of TRAP on [$^3$H]iloprost binding to platelet membranes and on [$^3$H]SQ29,548 binding to solubilized platelet membranes compared with their controls were performed using a two-sample Student's *t*-test (*$P < 0.05$).**

### *G-Protein Specificity of Inter-Receptor Signaling*

To investigate the specificity of this redistribution of receptor affinities, we conducted experiments to test the effect of thrombin receptor activation on ligand binding to a separate receptor population, i.e. $PGI_2$ receptors [21–24]. It might be expected that if inter-receptor signaling only occurs between receptors that couple to the same class of Gα subunits, there should be no G-protein signaling between these receptor classes, since thrombin receptors couple to $G\alpha_q$ [25, 26] and $PGI_2$ receptors couple to $G\alpha_s$ [23, 24]. To examine this notion, the effect of TRAP on the binding of the $PGI_2$ agonist iloprost [22, 23] to platelet membranes was measured. It was found that whereas TRAP produced a 50% increase in [$^3$H]SQ29,548 binding to $TXA_2$ receptors (Fig. 3, open bar), it had no significant effect on specific [$^3$H]iloprost binding to $PGI_2$ receptors (solid bar). These findings, therefore, provide evidence that thrombin receptors do not appear to modulate $PGI_2$ receptor affinity, presumably because they share different G-protein α-subunits, i.e. $G\alpha_q$ versus $G\alpha_s$.

On the other hand, our hypothesis would predict that $PGD_2$ receptors [19, 20] indeed should communicate with $PGI_2$ receptors, since they share a common G protein α-subunit, i.e. $G\alpha_s$. This possibility was tested in a separate series of experiments that measured iloprost binding to platelet membranes treated with either vehicle or $PGD_2$. It can be seen in Fig. 4 that 10 nM $PGD_2$ produced a significant increase (≈40%) in [$^3$H]iloprost specific binding. The specificity of this increase next was examined by measuring the effect of $PGD_2$ on SQ29,548 binding to $TXA_2$ receptors. However, it was found that unlike its

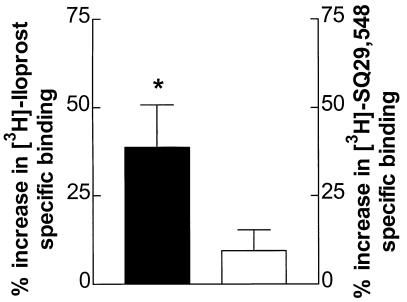


**FIG. 4. Effect of $PGD_2$ on [$^3$H]iloprost binding to platelet membranes and [$^3$H]SQ29,548 binding to solubilized platelet membranes. Platelet membranes and solubilized platelet membranes were pretreated with 10 nM $PGD_2$ for 3 min. Binding was performed as described under Materials and Methods, and the results are expressed as the percentage variation in [$^3$H]iloprost (solid bar) or [$^3$H]SQ29,548 (open bar) specific binding relative to unstimulated platelet membranes. Experiments were done at least seven times in triplicate, and the values represent means ± SEM of all results. The average [$^3$H]iloprost and [$^3$H]SQ29,548 specific binding values in the absence of agonist pretreatment were 65 ± 7 and 298 ± 40 fmol/mg protein, respectively. Statistical analyses measuring the effect of $PGD_2$ on [$^3$H]iloprost binding to platelet membranes and on [$^3$H]SQ29,548 binding to solubilized platelet membranes compared with their controls were performed using a two-sample Student's *t*-test (*$P < 0.05$).**

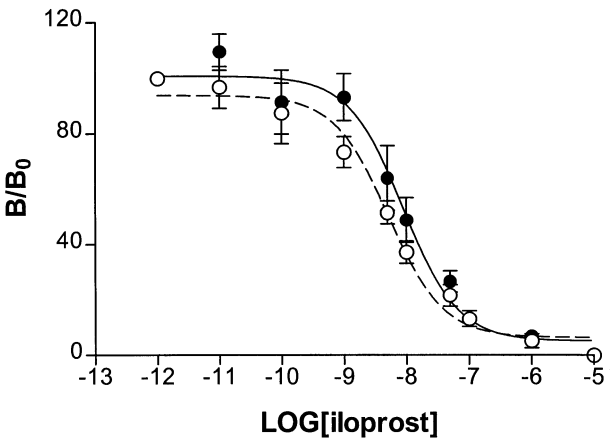


**FIG. 5. Effect of $PGD_2$ on the competition of [$^3$H]iloprost binding to platelet membranes. Platelet membranes were incubated with vehicle (●) or 10 nM $PGD_2$ (○) for 3 min, and [$^3$H]iloprost binding was measured in the presence of increasing concentrations of iloprost, as described under Materials and Methods. The percentage of specific binding is plotted as a function of added iloprost concentration. Each point represents the mean ± SEM of five separate experiments done in triplicate. B = [$^3$H]iloprost specific binding at a given iloprost concentration. $B_0$ = [$^3$H]iloprost specific binding in the absence of iloprost.**

effect on binding to $PGI_2$ receptors, 10 nM $PGD_2$ did not increase ligand binding to $TXA_2$ receptors significantly (Fig. 4, open bar).

Since the above results indicated that $PGD_2$ receptor activation could increase $PGI_2$ ligand binding, we next performed iloprost self-competition studies [23] to investigate the effect of $PGD_2$ on $PGI_2$ receptor affinity distribution. In these experiments, platelet membranes that were pretreated with either vehicle or 10 nM $PGD_2$ were incubated with [$^3$H]iloprost and increasing concentrations of iloprost. It can be seen in Fig. 5 that iloprost efficiently competed for its own binding in both vehicle- and $PGD_2$-treated membranes. In the absence of $PGD_2$, nonlinear regression analysis of the binding data favored a single binding site with an $IC_{50}$ of 10.1 ± 2.3 nM and a Hill coefficient of −0.80 ± 0.17 (N = 5). In contrast, when membranes were treated with $PGD_2$, a multi-site model was favored, i.e. the Hill coefficient shifted to −0.58 ± 0.10 (N = 5). On this basis, these latter binding data best fit a two-site model ($P < 0.05$), which described one binding site with an $IC_{50}$ of 3.3 ± 1.5 nM and the second binding site with an $IC_{50}$ of 12.5 ± 5.9 nM. These results, therefore, provide evidence that activation of $PGD_2$ receptors causes the appearance of new high-affinity $PGI_2$ receptors. In contrast, when platelet membranes were treated with 50 μM TRAP, no significant change in $IC_{50}$ (13.7 ± 3.7 nM, N = 3) or Hill coefficient (−0.86 ± 0.22, N = 3) could be detected. Taken together, these results demonstrate that inter-receptor signaling can occur between $PGD_2$ receptors and $PGI_2$ receptors, but not between thrombin receptors and $PGI_2$ receptors.

Since the above results demonstrated signaling between $PGI_2$ and $PGD_2$ receptors, experiments were performed to investigate a possible physiological consequence of such communication. In these experiments, the inhibitory effects of $PGI_2$ and $PGD_2$ were measured on TRAP-induced platelet aggregation [38, 39]. It was found (Fig. 6) that the combination of 1 nM $PGI_2$ with 1.5 nM $PGD_2$ (trace f) caused a greater inhibition than twice the concentration of either $PGI_2$ (2 nM, trace d) or $PGD_2$ (3 nM, trace e). This inhibition produced by combining $PGI_2$ and $PGD_2$, therefore, conforms to the rigorous definition of synergism that requires the biological effect caused by two agonists added together to exceed the response caused by twice the dose of either agonist added alone.

Taken together, the above results demonstrated that there is inter-receptor signaling between receptors that share a common G-protein but no such signaling between receptors that do not. This relationship has been shown for thrombin, $TXA_2$, and PAF [8] receptors, all of which couple to $G\alpha_q$ [5–28, 40]. It has also been shown in the present study for the two $G\alpha_s$-coupled receptors, $PGI_2$ and $PGD_2$.

### *Inter-Receptor Signaling in HPAEC*

The final series of experiments were performed to investigate whether the phenomenon of communication between seven-transmembrane receptors is limited to platelets or whether it occurs in other cell types as well. In the first experiments, SQ29,548 binding to intact HPAEC treated with either vehicle or TRAP was measured. Figure 7 illustrates that pretreatment with 50 μM TRAP caused a

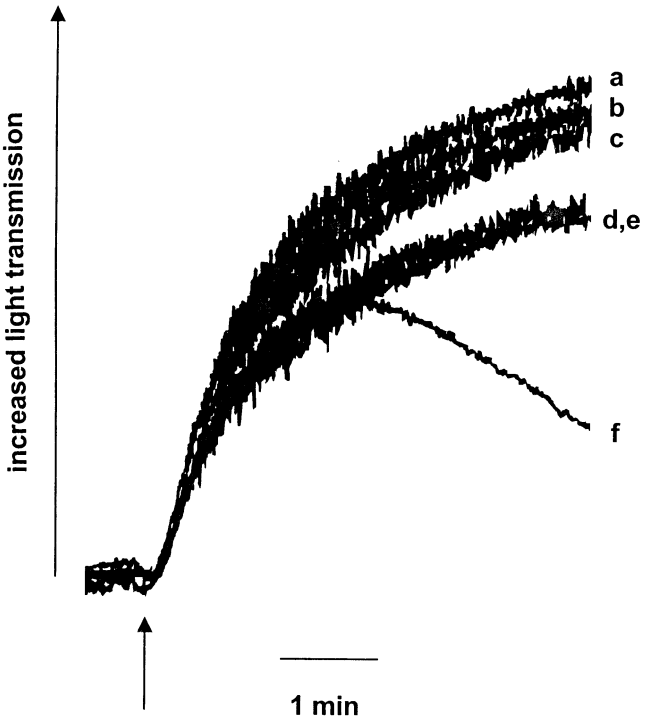


**FIG. 6. Synergistic inhibitory effect of $PGI_2$ and $PGD_2$ on platelet aggregation. Platelet-rich plasma was incubated for 1 min with vehicle (trace *a*), 1 or 2 nM $PGI_2$ (traces *b* and *d*, respectively), 1.5 or 3 nM $PGD_2$ (traces *c* and *e*, respectively), or 1 nM $PGI_2$ plus 1.5 nM $PGD_2$ (trace *f*), and aggregation was stimulated (at arrow) with 40 μM TRAP. The aggregation traces are representative of three separate experiments.**

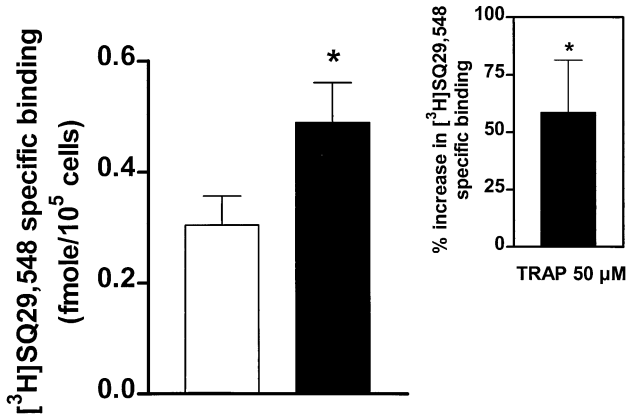


**FIG. 7. [$^3$H]SQ29,548 binding to intact HPEAC. HPEAC grown in modified MCDB 131 medium supplemented with 10% FBS were treated with vehicle (open bar) or 50 μM TRAP (solid bar) for 3 min. Binding was performed as described under Materials and Methods, and results are expressed as [$^3$H]SQ29,548-specific binding (fmol/$10^5$ cells). Experiments were done eight times in triplicate, and the values represent means ± SEM of all results. The average specific binding in the absence of agonist pretreatment was 0.34 ± 0.06 fmol/$10^5$ cells. Statistical analysis measuring the effect of TRAP on [$^3$H]SQ29,548 binding to HPEAC was performed using a two-sample Student's *t*-test (*$P < 0.05$). (Inset) Average TRAP-induced percentage increase in [$^3$H]SQ29,548 binding to HPEAC (*$P < 0.05$).**

substantial increase in [$^3$H]SQ29,548 specific binding. The average of eight such experiments revealed that the magnitude of this increase in [$^3$H]SQ29,549 specific binding was ≈60% (Fig. 7, inset).

To further elucidate the effects of thrombin receptor activation on endothelial cell $TXA_2$ receptor affinity distribution, we performed SQ29,548 competition-binding studies using the $TXA_2$ receptor agonist U46619 as a competing agent. In these experiments, HPAEC that were pretreated with either vehicle or 50 μM TRAP [9–12, 16–18] were incubated with [$^3$H]SQ29,548 and increasing concentrations of U46619. It can be seen in Fig. 8 that U46619 effectively competed for SQ29,548 binding in both vehicle- and TRAP-treated intact endothelial cells. In the absence of TRAP, nonlinear regression analysis of the binding data revealed a Hill coefficient of −0.25 ± 0.65 (N = 7), indicating the existence of multiple binding sites. This finding is consistent with the reported presence in endothelial cells of two separate $TXA_2$ receptor isoforms [41]. Upon TRAP treatment, there was no significant shift in the Hill coefficient, i.e. -0.25 (vehicle) versus -0.40 ± 0.40 (TRAP). However, it can be seen (Fig. 8) that TRAP did shift the competition-binding curve to the left, indicating that thrombin receptor activation caused an increase in $TXA_2$ receptor affinity for the agonist U46619. This increased affinity was evidenced by a significant ($P <$ 0.001) decrease in average $IC_{50}$ from 1.2 ± 0.5 μM ($K_i$ = 0.8 ± 0.3 μM) to 0.3 ± 0.1 μM ($K_i$ = 0.2 ± 0.1 μM), for vehicle- and TRAP-treated, respectively. These results, therefore, provide evidence that thrombin receptor activa-

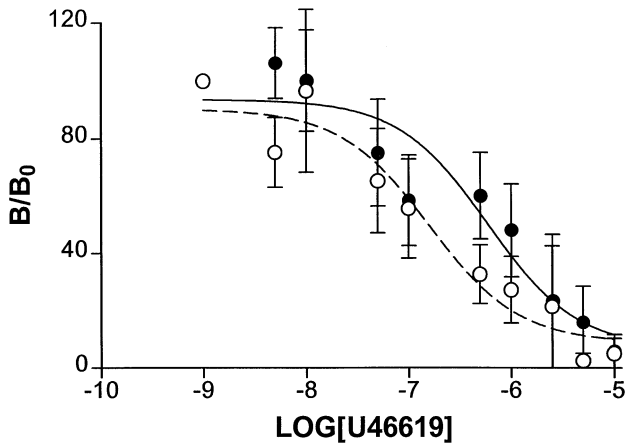


**FIG. 8. Effect of TRAP on the competition of [$^3$H]SQ29,548 binding to intact HPAEC by U46619. HPEAC grown in modified MCDB 131 medium supplemented with 10% FBS were treated with vehicle (●) or 50 μM TRAP (○) for 3 min, and [$^3$H]SQ29,548 binding was measured in the presence of increasing concentrations of U46619, as described under Materials and Methods. The percentage of specific binding is plotted as a function of added U46619 concentration. Each point represents the mean ± SEM of seven separate experiments done in triplicate. B = [$^3$H]SQ29,548 specific binding at a given U46619 concentration. $B_0$ = [$^3$H]SQ29,548 specific binding in the absence of U46619.**

tion causes a change in $TXA_2$ receptor affinity in endothelial cells. Whether this change in receptor affinity is due to the emergence of a new subpopulation of $TXA_2$ receptors or an increase of the affinity of all $TXA_2$ receptors is not known. However, these results are consistent with our findings in platelets indicating a redistribution of receptor affinities upon thrombin receptor activation.

## DISCUSSION

We have previously described a communication pathway, termed inter-receptor signaling, between platelet thrombin and $TXA_2$ receptors [8]. In this signaling process, activation of thrombin receptors causes increased $G\alpha_q$ coupling to $TXA_2$ receptors, shifting them to a higher affinity state. When fitted to a single binding site model, treatment with TRAP resulted in a 25% increase in average $TXA_2$ receptor affinity. This increased affinity, in turn, was used as a basis to explain the synergism that occurs during activation of these two separate receptors [8]. However, our earlier results did not differentiate between two possibilities, i.e. increased affinity for all $TXA_2$ receptors or increased affinity for a receptor subpopulation. This issue was investigated in the present report by performing competition-binding experiments in platelets using separate ligands, i.e. BM13.505 and U46619, as competing agents. It was found that with both ligands, TRAP caused a shift in the $TXA_2$ receptor binding profile from a single-site model (Hill coefficient equals $-1$) to a multi-site model (Hill coefficient equals $-0.48$). These findings therefore provide evidence that thrombin receptor activation induces a redistribution of affinities within the $TXA_2$ receptor pool, suggesting that $TXA_2$ receptors can exist in a dynamic equilibrium between single and multiple affinity states. The occurrence of such equilibrium is supported by growing evidence indicating that seven-transmembrane receptors coexist in at least two distinct states, i.e. high-affinity receptors that are G-protein coupled, and low-affinity receptors that are uncoupled [42–46]. Indeed, such a dynamic equilibrium may explain why it has been difficult to definitively establish whether $TXA_2$ receptor ligand binding follows a single or multi-site model. Therefore, depending on the ligands, the preparations, and the experimental conditions, different affinity profiles have been observed [34, 47–51]. A similar dynamic equilibrium presumably exists with another platelet receptor, i.e. the $PGI_2$ receptor. Thus, our results demonstrated that activation of $PGD_2$ receptors causes a redistribution of $PGI_2$ receptor affinities. In summary, it appears that the affinity distribution of seven-transmembrane receptors can be modulated by the activation of separate seven-transmembrane receptors.

We next investigated the specificity of inter-receptor signaling between different receptor types. Our proposed mechanism states that agonist activation of one receptor causes redistribution of its Gα subunit(s) to a different receptor class. Since it is also clear, however, that cells need to maintain a certain integrity in their separate signaling pathways, there must be a selection process by which Gα subunit redistribution is limited to certain receptor types. Based on this consideration, experiments were performed to investigate whether inter-receptor signaling only occurs between receptors that couple to the same Gα subunit. This was tested by evaluating communication between receptors that couple to $G_q$ and receptors that couple to $G_s$. It was found that whereas activation of $G\alpha_s$-coupled $PGD_2$ receptors does increase specific ligand binding to another $G\alpha_s$-coupled receptor ($PGI_2$ receptor), it does not increase ligand binding to $TXA_2$ receptors, which are $G\alpha_q$-coupled. Conversely, while TRAP increases binding to $TXA_2$ receptors, it does not increase binding to $G\alpha_s$-coupled receptors ($PGI_2$ receptor). Taken together, these results provide

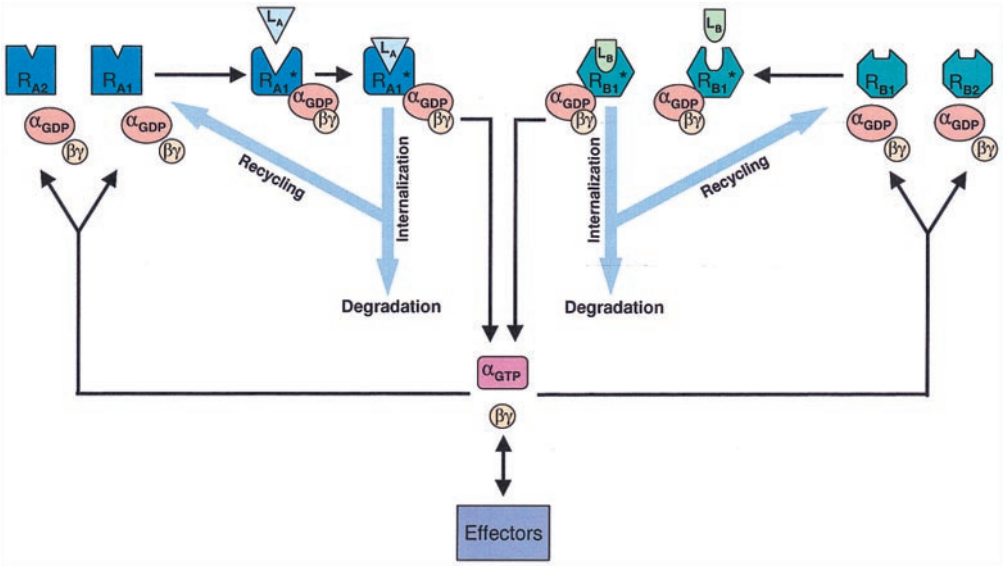


**FIG. 9. Inter-receptor G-protein signaling model. R, low-affinity receptor; R*, high-affinity receptor; L, receptor agonist. $R_A$ and $R_A$* represent one receptor class, $R_B$ and $R_B$* a separate one.**

evidence that inter-receptor signaling requires that receptors couple to a common family of G-proteins. The existence of such G-protein specificity would provide cells with a mechanism to integrate only those signaling pathways leading to similar intracellular effects. Thus, upon activation of a given receptor, redistribution of its associated G-proteins would sensitize other receptors that activate a common signal transduction cascade. For example, in normal hemostasis, low concentrations of thrombin could sensitize platelets to $TXA_2$ and thereby amplify the aggregation response at a site of vascular injury [52]. Alternatively, this signaling phenomenon may be involved in platelet sensitization between different agonists, e.g. thrombin, $TXA_2$, and PAF, in thromboembolic disorders [53, 54].

Collectively, these findings can be presented in a model (Fig. 9) to describe the dynamic equilibrium that may exist between receptors sharing common Gα subunits. In this model, receptors exist in two different states: uncoupled low affinity and G-protein-coupled high affinity. Upon agonist binding to high-affinity receptors, Gα subunits dissociate and interact with their effectors. The signal is terminated by GTP hydrolysis followed by reassociation of the Gα subunit, the Gβγ dimer, and a receptor. During this reassociation process, the heterotrimer may or may not recouple to its original receptor partner. Rather, mass action, i.e. the availability and affinity of a given receptor for the heterotrimer complex, will determine reassociation. This model, therefore, implies that there can be a cycling of G-proteins not only within a specific receptor class but also between receptors that share common Gα subunits. This cycling process would, in turn, provide a mechanism by which cells can respond to external perturbations, dynamically integrate separate signaling pathways that share common effector targets, and thereby reprioritize their physiological responsiveness.

In summary, the present results demonstrate in platelets that activation of one seven-transmembrane receptor can shift the affinity distribution of a separate seven-transmembrane receptor. Preliminary results suggest that endothelial cells may also share this adaptive mechanism.

*This work was supported, in part, by National Institutes of Health Grant HL-24350 and was conducted under the auspices of the Association for U.S.-French Biomedical Cooperation.*

## References

1. Stryer L, Molecular design of an amplification cascade in vision. *Biopolymers* **24:** 29–47, 1985.
2. Gilman AG, G-proteins: Transducers of receptor-generated signals. *Annu Rev Biochem* **56:** 615–649, 1987.
3. Wess J, G-protein-coupled receptors: Molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* **11:** 346–354, 1997.
4. Brass LF, Manning DR, Cichowski K and Abrams CS, Signaling through G protein in platelets: To the integrins and beyond. *Thromb Haemost* **78:** 581–589, 1997.
5. Hollingsworth EB, de la Druz RA and Daly JW, Accumulation of inositol phosphates and cyclic AMP in brain slices: Synergistic interactions of histamine and 2-chloroadenosine. *Eur J Pharmacol* **122:** 45–50, 1986.
6. Hill SJ and Kendall DA, Studies on the adenosine receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cerebral cortex. *Br J Pharmacol* **91:** 661–669, 1987.
7. Felder CC, Williams HL and Axelrod J, A transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding protein $G_i$ that amplifies ATP-mediated arachidonic acid release. *Proc Natl Acad Sci USA* **88:** 6477–6480, 1991.
8. Djellas Y, Antonakis K and Le Breton GC, A molecular mechanism for signaling between seven-transmembrane receptors: Evidence for a redistribution of G proteins. *Proc Natl Acad Sci USA* **95:** 10944–10948, 1998.
9. Vu T-KH, Hung DT, Wheaton VI and Coughlin SR, Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64:** 1057–1068, 1991.
10. Vu T-KH, Wheaton VI, Hung DT, Charo I and Coughlin SR, Domains specifying thrombin-receptor interaction. *Nature* **353:** 674–677, 1991.
11. Connolly AJ, Ishihara H, Kahn ML, Farese RV Jr and Coughlin SR, Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381:** 516–519, 1996.
12. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T and Coughlin SR, Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386:** 502–506, 1997.
13. Ushikubi F, Nakajima M, Hirata M, Okuma M, Fujiwara M and Narumiya S, Purification of the thromboxane $A_2$/prostaglandin $H_2$ receptor from human blood platelets. *J Biol Chem* **264:** 16496–16501, 1989.
14. Hirata M, Hayashi Y, Ushikubi F, Yokota 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.
15. Kim S-O, Lim CT, Lam SC-T, Komiotis D, Venton DL and Le Breton GC, Purification of the human blood platelet thromboxane $A_2$/prostaglandin $H_2$ receptor protein. *Biochem Pharmacol* **43:** 313–322, 1992.
16. Bahou WF, Coller BS, Potter CL, Norton KJ, Kutok JL and Goligorski MS, The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. *J Clin Invest* **91:** 1405–1413, 1993.
17. Lau L-F, Pumiglia K, Côté YP and Feinstein MB, Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem J* **303:** 391–400, 1994.
18. Seiler SM, Peluso M, Michel IM, Goldenberg H, Fenton JW II, Riexinger D and Natarajan S, Inhibition of thrombin and SFLLR-peptide stimulation of platelet aggregation, phospholipase $A_2$ and $Na^+/H^+$ exchange by a thrombin receptor antagonist. *Biochem Pharmacol* **49:** 519–528, 1995.
19. Siegl AM, Smith JB and Silver MJ, Specific binding sites for prostaglandin $D_2$ on human platelets. *Biochem Biophys Res Commun* **90:** 291–296, 1979.
20. Ito S, Negishi M, Sugama K, Okuda-Ashitaka E and Hayaishi O, Signal transduction coupled to prostaglandin $D_2$. *Adv Prostaglandin Thromboxane Leukot Res* **21:** 371–374, 1990.
21. Siegl AM, Smith JB, Nicolaou KC, Ahern S and Silver MJ, Selective binding site for [$^3$H]prostacyclin on platelets. *J Clin Invest* **63:** 215–220, 1979.

22. Steurer G, Jankovic B, Ettl K and Shernthaner G, Investigation of selective [$^3$H]iloprost binding sites on human platelet membranes. *Prog Clin Biol Res* **242:** 25–34, 1987.
23. Keen M, Kelly E and MacDermot J, Guanine nucleotide sensitivity of [$^3$H]iloprost binding to prostacyclin receptors. *Eur J Pharmacol* **207:** 111–117, 1991.
24. Negishi M, Hashimoto H and Ichikawa A, Translocation of α subunits of stimulatory guanine nucleotide-binding proteins through stimulation of the prostacyclin receptor in mouse mastocytoma cells. *J Biol Chem* **267:** 2364–2369, 1992.
25. Benka ML, Lee M, Wang G-R, Buckman S, Burlacu A, Cole L, DePina A, Dias P, Granger A, Grant B, Hayward-Lester A, Karki S, Mann S, Marcu O, Nussenzweig A, Piepenhagen P, Raje M, Roegiers F, Rybak S, Salic A, Smith-Hall J, Waters J, Yamamoto N, Yanowitz J, Yeow K, Busa WB and Mendelshon WE, The thrombin receptor in human platelets is coupled to a GTP binding protein of the $G\alpha_q$ family. *FEBS Lett* **363:** 49–52, 1995.
26. Verrall S, Ishii M, Chen M, Wang L, Tram T and Coughlin SR, The thrombin receptor second cytoplasmic loop confers coupling to $G_q$-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *J Biol Chem* **272:** 6898–6902, 1997.
27. Shenker A, Goldsmith P, Unson CG and Spigel AM, The G-protein coupled to the thromboxane $A_2$ receptor in human platelets is a member of the novel $G_q$ family. *J Biol Chem* **266:** 9309–9313, 1991.
28. Knezevic I, Borg C and Le Breton GC, Identification of $G_q$ as one of the G-proteins which copurify with human platelet thromboxane $A_2$/prostaglandin $H_2$ receptors. *J Biol Chem* **268:** 26011–26017, 1993.
29. Molino M, Woolkalis MJ, Reavay-Cantwell J, Pratico D, Andrade-Gordon P, Barnathan ES and Brass LF, Endothelial cell thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. *J Biol Chem* **272:** 11133–11141, 1997.
30. Kent KG, Collins LJ, Schwerin FT, Raychowdhury MK and Ware JA, Identification of functional $PGH_2/TXA_2$ receptors on human endothelial cells. *Circ Res* **72:** 958–965, 1993.
31. Garcia JGN, Patterson C, Bahler C, Aschner J, Hart CM and English D, Thrombin receptor activating peptides induce $Ca^{2+}$ mobilization, barrier dysfunction, prostaglandin synthesis, and platelet-derived growth factor mRNA expression in cultured endothelium. *J Cell Physiol* **156:** 541–549, 1993.
32. Raychowdhuri MK, Yukawa M, Collins LJ, McGrail SH, Kent KC and Ware JA, Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane $A_2$ receptor. *J Biol Chem* **269:** 19256–19261, 1994.
33. Ogletree ML, Harris DN, Greenberg R, Haslanger MF and Nakane M, Pharmacological actions of SQ29,548, a novel selective thromboxane antagonist. *J Pharmacol Exp Ther* **234:** 435–441, 1985.
34. Hedberg A, Hall SE, Ogletree ML, Harris DN and Liu EC-K, Characterization of [5,6-$^3$H]SQ 29,548 as a high affinity radioligand, binding to thromboxane $A_2$/prostaglandin $H_2$ receptors in human platelets. *J Pharmacol Exp Ther* **245:** 786–792, 1988.
35. Patscheke H, Stegmeier K, Hornberger W, Staiger Ch and Neugebauer G, Inhibition of platelet activation by the novel thromboxane receptor antagonist BM 13.505. *Thromb Haemost* **58:** 182, 1987.
36. Coleman RA, Humphrey PPA, Kennedy I, Levy GP and Lumley P, U-46619, a selective thromboxane $A_2$-like agonist? *Br J Pharmacol* **68:** 127p–128p, 1980.
37. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic activity. *Biochem Pharmacol* **22:** 3099–3108, 1973.
38. Hung SC, Ghali NI, Venton DL and Le Breton GC, Prostaglandin $F_{2\alpha}$ antagonizes thromboxane $A_2$-induced human platelet aggregation. *Prostaglandins* **24:** 195–206, 1984.
39. Born GVR and Cross MJ, The aggregation of blood platelets. *J Physiol (Lond)* **168:** 178–195, 1963.
40. Carlson SA, Chatterjee TK and Fisher RA, The third intracellular domain of the platelet-activating factor receptor is a critical determinant in receptor coupling to phosphoinositide phospholipase C-activating G proteins. Studies using intracellular domain minigenes and receptor chimeras. *J Biol Chem* **271:** 23146–23153, 1996.
41. Miggin SM and Kinsella BT, Expression and tissue distribution of the mRNAs encoding the human thromboxane $A_2$ (TP) α and β isoforms. *Biochim Biophys Acta* **1425:** 543–559, 1998.
42. Tian W-N, Duzic E, Lanier SM and Deth RC, Determinants of $\alpha_2$-adrenergic receptor activation of G proteins: Evidence for a precoupled receptor/G protein state. *Mol Pharmacol* **45:** 524–531, 1994.
43. Allan CJ, Higashiura K, Martin M, Morinelli TA, Kurtz DT, Geoffroy O, Meier GP, Gettys TW and Halushka PV, Characterization of the cloned HEL cell thromboxane $A_2$ receptor: Evidence that the affinity state can be altered by $G\alpha_{13}$ and $G\alpha_q$. *J Pharmacol Exp Ther* **277:** 1132–1139, 1996.
44. Clawges HM, Depree KM, Parker EM and Graber SG, Human 5-$HT_1$ receptor subtypes exhibit distinct G protein coupling behaviors in membranes from Sf9 cells. *Biochemistry* **36:** 12930–12938, 1997.
45. Barr AJ, Brass LF and Manning DR, Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor-G protein coupling. *J Biol Chem* **272:** 2223–2229, 1997.
46. Barr AJ and Manning DR, Agonist-independent activation of $G_z$ by the 5-hydroxytryptamine$_{1A}$ receptor co-expressed in *Spodoptera frugiperda* cells. Distinguishing inverse agonists from neutral antagonists. *J Biol Chem* **272:** 32979–32987, 1997.
47. Hung SC, Ghali NI, Venton DL and Le Breton GC, Specific binding of the thromboxane $A_2$ antagonist 13-azaprostanoic acid to human platelet membranes. *Biochim Biophys Acta* **728:** 171–178, 1983.
48. Mais DE, Burch RM, Saussy DL Jr, Kochel PJ and Halushka PV, Binding of a thromboxane $A_2$/prostaglandin $H_2$ receptor antagonist to washed human platelets. *J Pharmacol Exp Ther* **235:** 729–734, 1985.
49. Kattelman EJ, Venton DL and Le Breton GC, Characterization of U46619 binding to unactivated, intact human platelets and determination of binding site affinities of four $TXA_2/PGH_2$ receptor antagonists (13-APA, BM 13.177, ONO 3708 and SQ 29,548). *Thromb Res* **41:** 471–481, 1986.
50. Morinelli TA, Oatis JE Jr, Okwu AK, Mais DE, Mayeux PR, Masuda A, Knapp DR and Halushka PV, Characterization of an $^{125}$I-labeled thromboxane $A_2$/prostaglandin $H_2$ receptor agonist. *J Pharmacol Exp Ther* **251:** 557–562, 1989.
51. Dorn GW II, Distinct platelet thromboxane $A_2$/prostaglandin $H_2$ receptor subtypes. A radioligand binding study of human platelets. *J Clin Invest* **84:** 1883–1891, 1989.
52. Packham MA, Role of platelets in thrombosis and hemostasis. *Can J Physiol Pharmacol* **72:** 278–284, 1994.
53. FitzGerald DJ and FitzGerald GA, Role of thrombin and thromboxane $A_2$ in reocclusion following coronary thrombolysis with tissue-type plasminogen activator. *Proc Natl Acad Sci USA* **86:** 7585–7589, 1989.
54. White BP, Sullivan AT and Lumley P, Prevention of intracoronary thrombosis in the anaesthetised dog: The importance of thromboxane $A_2$ and thrombin. *Thromb Haemost* **71:** 366–374, 1994.