

Co-activation of Gi and Gq proteins exerts synergistic effect on human platelet aggregation through activation of phospholipase C and Ca²⁺ signalling pathways

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Abbreviations: 5-HT, 5-hydroxytryptamine; G protein, GTP binding protein; IP₃, inositol-1,4,5-trisphosphate; PKC, protein kinase C; NO, nitric oxide

Abstract

Our previous studies have shown that subthreshold concentrations of two platelet agonists exert synergistic effects on platelet aggregation. Here we studied the mechanism of synergistic interaction of 5-hydroxy-tryptamine (5-HT) and epinephrine mediated platelet aggregation. We show that 5-HT had no or little effect on aggregation but it did potentiate the aggregation response of epinephrine. The synergistic interaction of 5-HT (1-5 μ M) and epinephrine (0.5-2 μ M) was inhibited by α_2 -adrenoceptor blocker (yohimbine; IC₅₀= 0.4 μ M), calcium channel blockers (verapamil and diltiazem with IC₅₀ of 10 and 48 mM, respectively), PLC inhibitor (U73122; IC₅₀=6 μ M) and nitric oxide (NO) donor, SNAP (IC₅₀=1.6 μ M)). The data suggest that synergistic effects of platelet agonists are receptor-mediated and occur through multiple signalling pathways including the activation PLC/Ca²⁺ signalling cascades.

Keywords: platelet Aggregation, phospholipase C, calcium channel blockers, epinephrine, 5-hydroxytryptamine

Introduction

Platelets react rapidly with a wide range of agonists to maintain vascular integrity and haemostasis. Upon vascular damage, platelets undergo rapid transformation; become more spherical, extrude pseudopodia and

activate their fibrinogen receptors leading to aggregation. During this activation process, platelets release granule contents and substances which act in autocrine fashion to further enhance aggregation and haemostasis (Siess, 1989; Brass *et al.*, 1993). The platelet agonists act in synergy to potentiate each other's effect (Ware *et al.*, 1987).

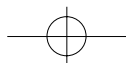
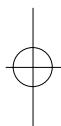
Most of the platelet agonists, like thrombin, ADP, PAF, epinephrine and 5-HT, interact with their transmembrane receptors on platelets coupled with GTP binding proteins (G proteins). The G-proteins mediate a variety of cellular processes by activating different effector molecules, including adenylyl cyclase, inositol phospholipid-specific phospholipase C (PLC) or ion channels (Siess *et al.*, 1989; Exton, 1996). In human platelets, activation of Gs/adenylyl cyclase decreases platelet aggregation. In contrast, activation of Gi/adenylyl cyclase (e.g. by epinephrine) leads to decrease intracellular cAMP levels and increases platelet aggregation (Siess *et al.*, 1989). In platelets, stimulation of receptors coupled with phosphoinositidase C-linked G-proteins (Gq) (e.g., by 5-HT, PAF or thrombin) leads to activation of PLC and thus generation of second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃). This results in the activation of protein kinase C (PKC) and the mobilization of intracellular Ca²⁺, respectively [Obberghen-Schilling and Pouyssegur, 1993]. Both Ca²⁺ and PKC act in synergy to enhance (Crabos *et al.*, 1992) whereas deficiency of Gq protein leads to impairment of platelet aggregation (Offermans *et al.*, 1997).

We and others have shown that subthreshold concentrations of platelet agonists potentiate aggregation response and thus exhibit synergistic effects (Ware *et al.*, 1987; Saeed *et al.*, 1997). This synergism is considered to occur mainly through priming of Ca²⁺ responses, but the role of other effectors and second messengers is not well known. Here we show that synergistic interaction of 5-HT and epinephrine can be inhibited by Ca²⁺-channel blockers and PLC inhibitor suggesting the involvement of calcium and Gq/PLC pathways in this cascade.

Materials and Methods

Chemicals

Epinephrine, 5-HT, diltiazem, verapamil and yohimbine were purchased from the Sigma Chemical Co. (St. Louis, MO. USA). U73122 and SNAP were from Alexis LC. Labs (UK). Antisera to various G proteins were kind gift



from Professor Graeme Milligan (Glasgow, UK). All other chemicals were of the highest purity grade available.

Preparation of human platelets

Blood was taken by veinpuncture from normal human volunteers reported to be free of medication for one week. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 *g* for 15 min at 20°C to obtain platelet rich plasma (PRP) with platelet counts between 2.5 and 3×10⁸/ml of plasma. All experiments were performed within 2 h of PRP preparation.

Measurement of platelet aggregation

Aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, U.S.A.) using 0.45 ml aliquots of PRP (Shah and Saeed, 1995). The final volume was made up to 0.5 ml with test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. Aggregation was induced with epinephrine and sub-threshold concentration determined. To obtain the synergistic effect of 5-HT and epinephrine, we added low concentrations of these agonists together. The anti-aggregatory effects were studied by pretreatment of PRP with various inhibitors for one minute followed by addition of the subthreshold concentrations of epinephrine and 5-HT. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. Once the anti-platelet activity of various inhibitors against agonists was established, dose-response curves were constructed to calculate the IC₅₀ values of the test substances. Statistical analysis was done using Student's *t*-test.

Western blots

The generation and specificities of the various antisera used in this study are already described (Mitchell *et al.*, 1991). Membrane samples were resolved by SDS/PAGE in 10% (w/v) acrylamide gels overnight at 60 V. For resolving Gqα and G11α, urea linear gradient gels were prepared and run overnight at 100 V as described in detail previously (Shah and Milligan, 1994). Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 h in 5% (w/v) gelatin in phosphate buffered saline (PBS), pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P-40 (NP-40) and incubated overnight. The primary antiserum was removed and blots washed extensively with PBS containing 0.2% NP-40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; HRP) in 1% gelatin/PBS/0.2% NP-40 was added and left for 3h. After removal of the second antiserum, blots were washed extensively as

above and developed with *o*-dianisidine hydrochloride as substrate for HRP.

Results and Discussion

Platelet membranes were subjected to immunoblot analysis using antisera specific against the α-subunit of Gs, Gi and Gq/11 proteins. Western blots results in Figure 1A show that human platelets express predominantly 45 kD adenylyl cyclase-stimulatory G protein (G_sα) as well as inhibitory (Gi2) G proteins. Using urea gradient gels under the conditions where Gqα and G11α proteins can be separated and detected by CQ2 antisera (Shah and Milligan, 1994), we find that platelets contain only Gqα protein (42 kD) but not G11α (Figure 1B). Normally Gqα and G11α are co-expressed in most of the body tissues (Mitchell *et al.*, 1991; Shah and Milligan, 1994).

Treatment of PRP with 5-HT up to 10 μM had no appreciable effect on platelet aggregation, however,

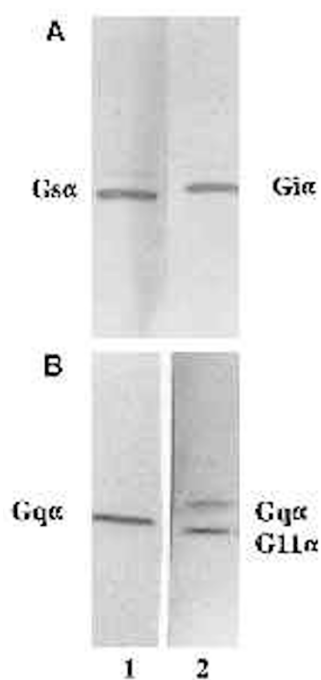


Figure 1. Immunoblot analysis of G proteins in membranes prepared from human platelets. (A): Detection of α-subunit of Gs and Gi proteins. (B): Detection of Gqα and G11α proteins after their separation by urea gradient gels. The antiserum CQ2 recognizes α-subunit of both Gqα and G11α proteins (Shah and Milligan, 1994). Platelets express only Gqα protein (lane 1) compared to both Gqα and G11α in other body tissues. Lane 2 shows Gqα and G11α in pituitary gonadotrophs.

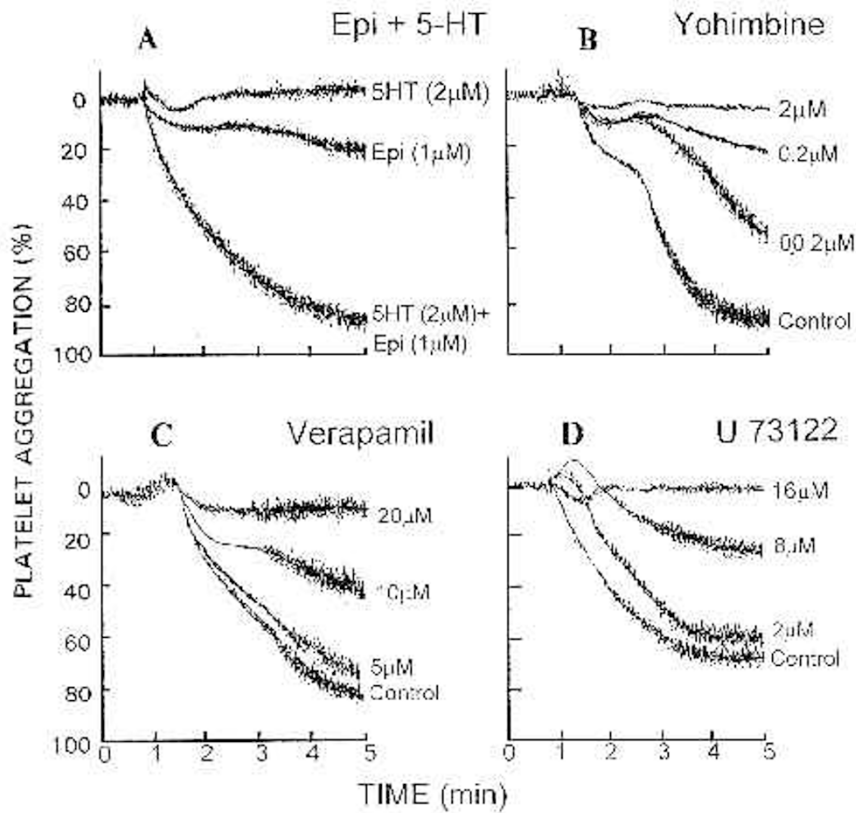


Figure 2. Effect of various inhibitors on platelet aggregation mediated by synergistic interaction of 5-HT and epinephrine. (A) Data from a representative experiment showing synergism of 5-HT (2 μM) and epinephrine (1 μM) which is inhibited by yohimbine (B), verapamil (C) and U73122 (D). Control means platelet aggregation induced by 5-HT (1-2 μM) plus epinephrine (0.5-2 μM).

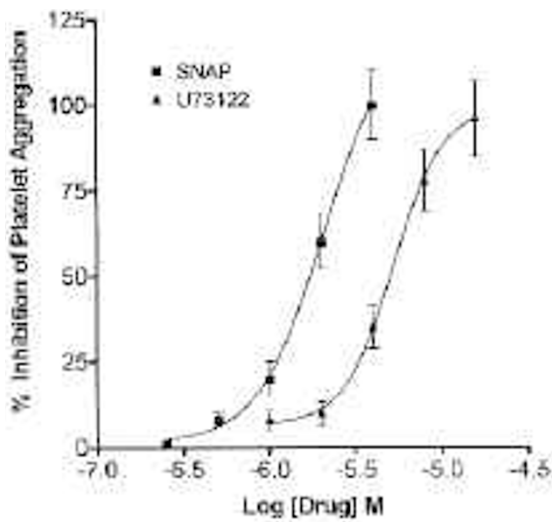


Figure 3. Dose-response inhibitory effect of NO donor, SNAP and phospholipase C inhibitor, U73122 on platelet aggregation induced by subthreshold concentrations of 5-HT (1-2 μM) and epinephrine (0.5-2 μM). Data is mean ± SEM (n=6). Both U73122 (2-16 μM) and SNAP (0.1-4 μM) showed dose-dependent significant inhibitory effect on aggregation.

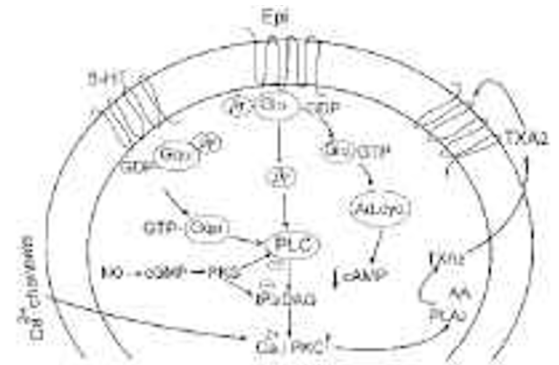


Figure 4. A model depicting the role of Gq and Gi proteins during co-activation by 5-HT and epinephrine (Epi). The $G_{q\alpha}$ and $G_{i\beta\gamma}$ subunits converge at phospholipase C (PLC), which leads to generation of inositol triphosphate (IP₃) and diacylglycerol (DAG) and thus release of Ca²⁺ from internal stores and activation of protein kinase C (PKC). Both Ca²⁺ and PKC are considered to be involved in the release of granule contents and activation of phospholipase A₂, thus generation of potent agonist, thromboxane A₂ (TXA₂) which in an autocrine fashion acts on platelets through Gq protein. Also Epi-mediated decrease in cAMP levels stimulate aggregation. NO from exogenously added SNAP (NO donor) inhibits platelet aggregation through production of cGMP from guanylate cyclase. The cGMP activates PKG which can phosphorylate PLC, Ca²⁺ channels or IP₃-receptor to inhibit Ca²⁺-mediated pro-aggregatory effects in platelets.

Table 1. Effect of various inhibitors on platelet aggregation mediated by synergistic interaction of 5-HT (1-5 μ M) and epinephrine (0.5-2 μ M). Data is mean \pm SEM (n=6-7) and is indicated as half-maximal effect (IC₅₀) of inhibitors.

Inhibitor	IC ₅₀ of inhibitor (mM)
Yohimbine	0.4 \pm 0.06
Verapamil	10 \pm 2
Diltiazem	48 \pm 8
U73122	6 \pm 0.8
SNAP	1.6 \pm 0.02

epinephrine (0.2-20 μ M) had concentration-dependent increase in platelet aggregation. The simultaneous addition of subthreshold concentrations of 5-HT (1-2 μ M) and epinephrine (0.5-1 μ M) exhibited synergistic effect (Figure 2A). 5-HT is considered a weak platelet agonist as it causes only shape change in platelets (Steen *et al.*, 1993), however, subthreshold concentrations of 5-HT and epinephrine, when used together, can elicit a strong synergistic effect on platelet aggregation. Such a mechanism of synergism is known among other agonists too and is considered to occur due to activation of Ca²⁺ signalling cascade. It has been reported that a rise in Ca²⁺ induced by first agonist primes platelets for an enhanced functional response to the second agonist (Ware *et al.*, 1987). Results show that the synergism between 5-HT and epinephrine is inhibited by pretreatment of PRP with yohimbine indicating that the effect is mediated through α_2 -adrenoceptors (Figure 2B). The half-maximal inhibitory concentration (IC₅₀) of yohimbine against 5-HT plus epinephrine was 0.4 μ M.

Receptors for 5-HT in platelets are coupled with Gq/PLC (De Chaffoy de Courcelles *et al.*, 1987; Martin, 1994) whereas epinephrine interacts with α_2 -adrenoceptors coupled with Gi/adenylyl cyclase pathway (Steen *et al.*, 1993). Recent studies show that $\beta\gamma$ -subunits of activated Gi protein can also activate PLC (Clapham and Neer, 1997; Banno *et al.*, 1998). We used PLC inhibitor (U73122) to examine if 5-HT and epinephrine mediated effects involved PLC. Results show that pretreatment of PRP with U73122 inhibits the platelet aggregation mediated by synergistic effect of 5-HT and epinephrine (Figure 2D). The IC₅₀ of U73122 was 6 μ M (Figure 3). This raises the possibility of convergence of Gi and Gq pathways at PLC during platelet aggregation. A similar phenomenon has been observed in HEK 293 cells where it is shown that G $\beta\gamma$ subunit-mediated α_2 -adrenoceptor and Gq/11 mediated α_{1B} -adrenoceptor-coupled MAP kinase pathways converge at the level of PLC (Della Rocca *et al.*, 1997). In fact the $\beta\gamma$ -subunits of Gi2 and Gq proteins have similar efficacy in regulation of effector (PLC) in human platelets (Banno *et al.*, 1998).

Since activation of Gq/PLC pathway leads to an increase in cytosolic Ca²⁺ due to its release from internal stores by IP₃ or through store-depleted calcium influx (Berridge, 1993; Heemskerk and Sage, 1994), we used Ca²⁺ channel blockers (verapamil and diltiazem) to see if the Ca²⁺ signalling is involved in aggregation following co-activation by 5-HT and epinephrine. We found that the synergistic effect of 5-HT and epinephrine is inhibited by both verapamil (IC₅₀= 10 μ M, Figure 2C) and diltiazem (IC₅₀= 48 μ M, Table 1). Verapamil has been shown to inhibit platelet aggregation, Ca²⁺ fluxes and thromboxane production induced by ADP, collagen and thrombin (Brocchieri *et al.*, 1995; Saeed *et al.*, 1997). It is well known that Ca²⁺ plays pivotal role in platelet aggregation (Heemskerk and Sage, 1994; Shah *et al.*, 1996, 1998). A rise in cytosolic Ca²⁺ levels accompanies platelet activation through stimulation of the enzymes which are not fully functional at low Ca²⁺ concentration present in the resting platelets (Heemskerk and Sage, 1994). Interruption in the process of Ca²⁺ activation either through Ca²⁺-channels (Brocchieri *et al.*, 1995; Shah *et al.*, 1997) or Gi/Gq proteins (Offermans *et al.*, 1997) can interfere in the activation of platelets.

Previously it was shown that cyclic nucleotides, cAMP and cGMP through activation of cAMP- and cGMP-dependent protein kinase, down-regulate the Ca²⁺ responses and thus inhibit platelet aggregation. We tested if increasing intracellular nitric oxide (NO) levels by NO donor (SNAP) and thus activating cGMP kinase has any inhibitory effect on platelet aggregation induced by epinephrine and 5-HT. Results show that SNAP inhibits platelet aggregation at very low concentrations (IC₅₀; 1.6 μ M) suggesting that epinephrine and 5-HT synergism is sensitive to NO generation (Figure 3). In fact platelets contain an abundance of cAMP and cGMP-dependent protein kinases (El-Daher, *et al.*, 1996) which can inhibit PLC-induced inositol phosphate production and inactivate IP₃ and thromboxane receptors (see review by Heemskerk and Sage, 1994; Wang *et al.*, 1998), thus inhibiting the platelet aggregation.

Multiple studies have shown that agents (like epinephrine) which decrease intracellular cAMP levels stimulate platelet aggregation (Brass *et al.*, 1993; Siess *et al.*, 1993). In fact epinephrine effects in platelets may involve multiple signalling pathways; stimulation of IP₃ production through stimulation of PLC by G protein $\beta\gamma$ -subunits (Clapham and Neer, 1997; Banno *et al.*, 1998), increase in Ca²⁺-influx, thus activation of phospholipase A2 (Heemskerk and Sage, 1994) and activation of some other proteins like Syk (Wang *et al.*, 1997). In conclusion, our results point to a new facet of 5-HT and epinephrine synergistic interaction and further demonstrate that such an effect is receptor mediated and occurs due to activation of Gq and Gi proteins which converge at PLC and Ca²⁺ signalling pathways as depicted in Figure 4.

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Erratum

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The authors would like to amend the name of Y. Yaqub¹ to M. Y. Yakoob¹.

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