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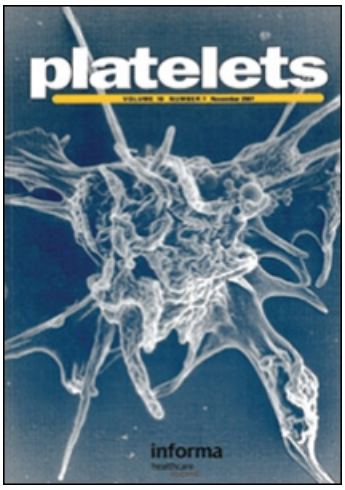

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ORIGINAL ARTICLE

Investigating GABA and its function in platelets as compared to neurons

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Abstract
We have recently suggested that platelets could be used as a model for neuronal receptors. In this paper we have investigated γ-aminobutyric acid (GABA) metabolism and GABA receptors in platelets and in cultured neurons to see whether platelets' GABA mimics neuronal GABA receptor activities. We used the ELISA technique for detecting the GABA concentration in platelet rich plasma and cultured neurons. The functional effects of GABA and its receptor ligands on platelets were determined using an aggregometer. We found that the GABA concentration is 30% lower in platelets than in neurons and in both preparations GABA was metabolized by GABA transaminase (GABA-T). GABA potentiated calcium dependent platelet aggregation with a higher value in washed platelets suspension (WPS) then in platelet rich plasma (PRP). This effect was inhibited by benzodiazepines, calcium channel blockers and the selective phosphoinositide 3-kinase antagonist Wortmannin. GABA neurotransmission is involved in most aspects of normal brain function and can be perturbed in many neuropathologic conditions. We concluded that platelets could be further developed to be used as a peripheral model to study neuronal GABAergic function and its abnormality in diseases such as epilepsy and schizophrenia. Furthermore our results indicated that PI3-kinase is involved in calcium dependent GABA induced platelet aggregation as this synergistic effect is inhibited by Wortmannin in dose dependent manner.

Keywords: *GABA, benzodiazepine, GABA-transaminase, PK11195, calcium ionophore A23187, Wortmannin, platelet aggregation*

Abbreviations: *GABA, γ-aminobutyric acid; PRP, platelet rich plasma; WPS, washed platelet suspension; ELISA, enzyme-linked immunosorbent assay; GABA-T, GABA-transaminase; TKL, tyrosine kinase-linked receptors; PI 3-K, phosphoinositide 3-kinase; PIP-3, phosphatidyl inositol 3,4,5-trisphosphate*

Introduction

γ-Aminobutyric acid (GABA) has been studied extensively as a central nervous system inhibitory neurotransmitter. However, it also appears in peripheral tissues where its functions are more varied and less completely understood [1]. For example, it has been known for many years that GABA is present in blood [2]; however, it is unclear whether it exerts any physiological effects on the components of blood, such as platelets. A number of other transmitter receptors are found in platelets [3, 4]. Platelets have been shown to possess a high affinity GABA uptake system sensitive to temperature and sodium ion concentration. Specific Na^+-independent GABA and muscimol binding to platelets has been observed. Benzodiazepine binding has also been observed in platelets [5–7], although not all neuronal GABA receptors contain the benzodiazepine binding γ subunit [8]. This indicates the presence of $GABA_A$ receptors although the subunits responsible have not been identified.

In this report, we investigated the possible functional effects of GABA in human platelets. As well as having relevance to potential effects of peripheral GABA, we also sought to establish the feasibility of using platelets as a model of neuronal GABA receptors. We focused on aggregation of platelets induced by the Ca^{2+} ionophore, A23187 [9] as our previous studies had revealed effects on aggregation by other transmitters and the role of multiple signalling pathways [10]. Aggregation was measured

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in the absence and presence of wortmannin, a mold sterol and potent inhibitor of PI 3-kinase [11]. We hypothesized that GABA receptor responses are present both in platelets and neurons and there could be a synergistic effect of GABA and calcium ionophore A23187 associated with platelet aggregation.

Materials and methods

Materials

Most drugs and chemicals were obtained from Sigma Chemical Co (St, Louis, MO, USA). All other chemicals were of the highest purity grade available and from suppliers as specified.

Preparation of human platelets

Blood was taken by venupuncture from normal human volunteers reported to be free of medication for one week. Blood samples were mixed with 3.8% sodium citrate solution (9 : 1) and centrifuged at 260 g for 15 min; the buffy coat fraction was separated and centrifuged, filtered to get platelet rich plasma (PRP) and further washed and suspended in buffer for washed platelets suspension (WPS).

The aggregation studies were carried out at 37°C with PRP, having platelet counts between 2.5 and 3.0×10^8/ml of plasma. This study was approved by the Human and Animal Research Ethic Committee, United Arab Emirate University Al Ain, UAE.

Preparation of cultured neurons

Hippocampal and cortical neuronal cultures were prepared according to the procedure described earlier [12]. Briefly, one to five-days-old rat pups were quickly decapitated. The hippocampi and cortex were removed using toothpicks and placed in 3 ml ice-cold Puck's saline. The tissues were mechanically dissociated by gentle trituration through a narrow-bored fire-polished Pasteur pipette with 30–40 passages in 3 ml cold Puck's saline (osmolality 330 mOsm and pH 7.4). One milligram of tissue yielded approximately 10^7 cells. Usually 4×10^5 viable cells were distributed into poly-L-lysinated air-dried 35-mm plastic plates containing 2 ml of freshly prepared modified EMEM (GIBCO-BRL, CA, USA; Invitrogen Corp., Karachi, Sind), 86 ml EMEM supplemented with 10 ml fetal calf serum, 1 ml L-glutamine, 2 ml penicillin/streptomycin and 1 ml fungizone. The plates were then incubated at 37°C in an atmosphere of humidified air with 5% CO_2 for up to two weeks.

Measurement of GABA levels by enzyme-linked immunosorbent assay (ELISA)

GABA concentrations in cultured neurons and platelets were measured by competitive ELISA technique according to the manufacturer's instructions (IBL Immuno-Biological Laboratories, Hamburg, Germany). Briefly, GABA in samples and controls was acylated with acetic anhydride in acetone and placed into 96-well microtitre plates coated with goat anti-rabbit IgG. Biotinylated GABA and rabbit GABA antiserum were added to each well and incubated overnight at 40°C. Paranitrophenylphosphate in a diethanolamine solution was used as a substrate following the application of alkaline phosphatase conjugated goat anti-biotin antibody. Samples were read at 405 nm on an ELISA plate reader (Molecular Devices, Union City, CA, USA). The amount of GABA was quantified using the standards supplied by the manufacturer and analysed using Origin software (Microcal Software, Northampton, MA, USA). GABA-transaminase (GABA-T, 100 nM), gabaculin (GABA-T antagonist, 0.1 μM), flunitrazepam (1 μM), and/or the peripheral benzodiazepine antagonist PK11195 (0.5 μM) were incubated with the cells for 12 hours, before taking the readings using an ELISA plate reader. Values were expressed as means ± SEM ($n = 7$) and statistical significance was determined using student *t*-test against control.

Measurement of platelet aggregation

Platelet aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation; Chicago, IL, USA) using 0.45 ml aliquots of PRP and WPS as described previously [13, 14]. The final volume was made up to 0.5 ml with sodium chloride (0.9%, w/v) or test drugs and incubated for 1 min before adding the GABA (4 μM) and/or the calcium ionophore, A23187 (0.5 μM). Aggregation was recorded for the subsequent 5 minutes for both combined and individual effects of GABA and A23187.

Results

γ-Aminobutyric acid (GABA) concentration was detected using the ELISA technique in both cultured neurons and platelets. GABA-T (100 nM) significantly decreased the concentration of GABA and this effect was blocked by the GABA-T antagonist gabaculine (0.1 μM).Flunitrazepam (1 μM) decreased the GABA concentration both in cultured neurons and platelets indicating the benzodiazepine binding sites in both preparations may have functional connotation. This inhibitory effect was significantly increased in platelets in the presence of (0.5 μM) peripheral benzodiazepine antagonist PK11195 (Table I and histogram).

GABA alone had no effect on human platelet aggregation up to a concentration of 50 μM; however, the calcium ionophore A23187 produced aggregation in a dose dependent manner. Treatment of platelets

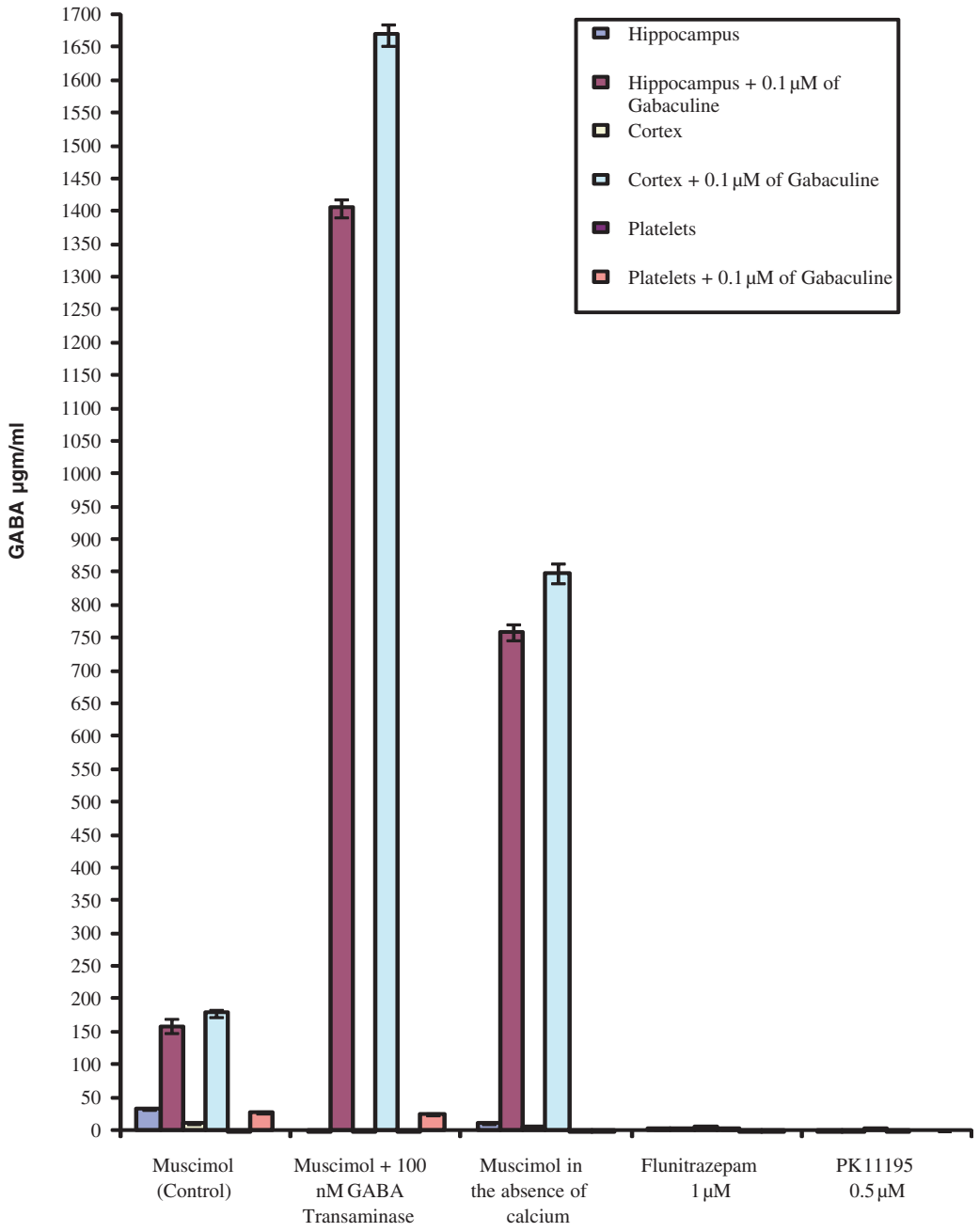

Table I. GABA concentration (μg/ml) in cultured cortical and hippocampal tissues and platelets.

Conditions	Hippocampus	Hippocampus + 0.1 μM of Gabaculine	Cortex	Cortex + 0.1 μM of Gabaculine	Platelets	Platelets + 0.1 μM of Gabaculine
Control	33 ± 1.4	160 ± 10.5	12 ± 0.3	180 ± 6.2	1.1 ± 0.02	28 ± 1.3
100 nM	0.30 ± 0.01	1406 ± 13.6	0.16 ± 0.02	1670 ± 16	0.05 ± 0.007	26 ± 1.3
GABA Transaminase	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.01$	
In the absence of calcium	13 ± 0.9	760 ± 12	6 ± 0.09	850 ± 15	0.3 ± 0.01	0.41 ± 0.02
						$P<0.001$
1 μM flunitrazepam	4 ± 0.1	3 ± 0.5	6 ± 0.9	4.5 ± 0.8	0.02 ± 0.001	0.033 ± 0.01
PK11195	2.4 ± 0.2	1.8 ± 0.09	3.1 ± 0.09	2.1 ± 0.1	0.0001 ± 0.00006	0.00009 ± 0.000001
0.5 μM					$P<0.001$	$P<0.001$

The effects of various stimuli on GABA concentrations (μg/ml) in neurons and platelets. Gabaculine (0.1 μM) potentiated the GABA concentrations but had no effect on benzodiazepine. Data are means ± SEM ($n=5$). $P<0.001$ *vs.* control.
Histogram 1. The data used to make this histogram is taken from Table I. X-axis shows the different stimuli used in both hippocampal and cortical neurons and in platelets to see their effect on GABA release Y-axis. Note that 0.1 μM Gabaculine in the presence of muscimol and GABA transaminase not only abolished GABA-T effect but produces a many fold increase in GABA concentration in both platelets and neurons. Data are means ± SEM ($n=5$). Top panel shows the histogram version of table I.

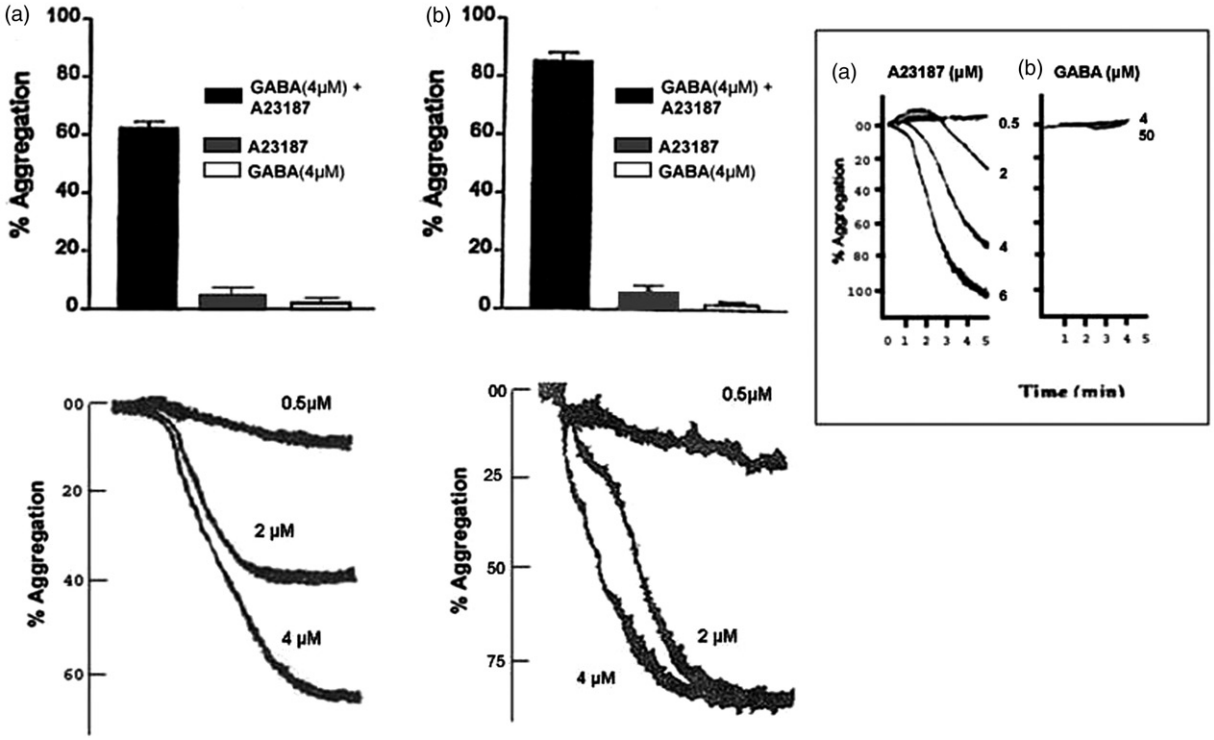

Figure 1. Effect of GABA (4 μM) + A23187 (0.5 μM), alone and in combination, on human platelets aggregation in platelet rich plasma [PRP] (a) and in washed platelet suspension [WPS] (b). Top panel: histograms; bottom panel: concentration response of A23187 in the presence of 4 μM GABA for both preparations. Data represent mean ± S.E.M. ($n = 5$). Inset: the percentage of aggregation in different concentrations of A23187 and GABA for (WPS). GABA alone in different concentrations was also not able to produce any aggregation in PRP (data not shown).

with a subthreshold concentration of A23187 (0.5 μM) in combination with a low concentration of GABA (4 μM) resulted in a marked potentiation of aggregation and was observed to be lower in PRP compared to WPS**.** The percentage for platelet aggregation increased from 0% in 0.5 μM A23187 to 10% in combination with 4 μM GABA in PRP, whereas from 0% to 20% under similar conditions in WPS. Both in PRP and WPS 100% aggregation was observed in 6 μM A23187 only. Thus the concentrations of A23187 used for the synergistic effect was below 6 μM. Maximum aggregation were 65% (PRP) and 85% (WPS) as a result of combined effect of 4 μM A23187 plus 4 μM GABA in both preparations (see Figure 1). A range of benzodiazepines inhibited aggregation**,** while bicuculline, picrotoxin and 5-aminovalerate, frequently used blockers of neuronal GABA receptors, were ineffective (up to 100 μM) as inhibitors of the aggregatory response evoked by GABA plus A23187. The calcium channel blockers, verapamil and diltiazem both showed inhibition with IC_{50} values of 7.5 ad 9 μM, respectively. Similarly, benzodiazepines, diazepam and flunitrazepam inhibited this synergistic effect with IC_{50} values of 25 and 7 μM, respectively. The specific inhibitor of PI 3-kinase, Wortmannin (10 nM), completely inhibited the synergistic interaction of GABA and A23187 (Figure 2 and Table II).

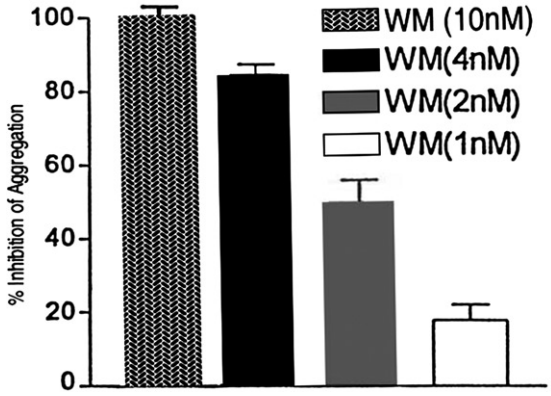

Figure 2. Inhibitory effect of Wortmannin on platelet aggregation induced by GABA (4 μM) + A23187 (0.5 μM). Data is mean ± S.E.M. ($n = 5$) $P < 0.001$

Discussion

In this report we addressed two questions: whether the metabolism of GABA is similar in both preparations and what is its role in platelet aggregation. Platelets are widely used to unravel thrombopathies and other human disorders [25] and we are focusing on developing platelets as peripheral model for neuronal disorders.

Table II. Inhibition of platelet aggregation by various agents.

Drugs	V_{max}	IC_{50} (μM) ± SEM	Hill coefficient
Diazepam	113 ± 7.6	25 ± 2.4	1.7
Flunitrazepam	32 ± 3.1	7 ± 4.3	2.5
Diltiazem	47 ± 2.9	9 ± 1.6	2.1
Verapamil	41 ± 3.6	7.5 ± 2.2	1.16
Wortmannin	10 ± 1.01	0.002 ± 0.001*	3.1

Concentration response data for the inhibition of platelet aggregation produced by synergistic effect of 4 μM GABA and 0.5 μM A23187. Data are means ± SEM ($n = 5$).

We measured GABA concentration in lysates of both cultured neurons and platelets using ELISA technique. An inhibitory effect of GABA transaminase on GABA in both preparations confirmed the earlier observations that the expression [23] and activity of GABA-T in platelets reflects the brain GABA-T activity [15]. Reduction of GABA release by GABA-T was not only abolished in the presence of gabaculine but the level of GABA was also increased (500 fold in platelets and 5000 fold in hippocampus neurons). This confirms the presence of endogenous GABA-T activities in both preparations. Smaller concentrations of GABA were detected in the presence of flunitrazepam in both preparations. For platelets, the GABA concentration was completely abolished by the peripheral benzodiazepine receptor blocker PK11195 and could be due to substrate negative feed back inhibition. Substrate induced modulation of neurotransmitter uptake is found to be present in human platelets [26].

We next investigated possible functional effects of GABA in platelets and found that GABA could potentiate more aggregation of platelets induced by the calcium ionophore A2187 in WPS as compared to PRP indicating a negative role of plasma proteins such as albumin and hepatoglobin in the process of aggregation. GABA and low doses of the calcium ionophore had no effect alone, but when applied together induced marked aggregation. There is little direct evidence that functional GABA receptors of the A or B types are normally present on platelets, although the degradative enzyme, GABA-transaminase, is found in the cytoplasm [16]. However, high affinity binding sites for GABA have been found to be present in the platelet membrane [2]. Addition of A23187, a lipophilic calcium ionophore, brings about rapid phosphorylation of numerous membrane proteins [17], presumably including those associated with receptors, binding sites, and ion channels. GABA binding may enhance the inward passage of calcium ions by an effect on ionophore-phosphorylated lipoprotein complexes – or by an enhanced Ca^{2+} electrochemical gradient. However, putative binding antagonists, bicuculline or 5-aminovaleric acid [18] were without effect in this study. The distinction between the proposed GABA binding site and a true $GABA_A$ receptor is further underscored by the effects on aggregation observed with diazepam and flunitrazepam. By acting on the gamma subunit in the $GABA_A$ receptor complex on neurons, benzodiazepines normally enhance the depressant effects of the transmitter. In addition, peripheral type benzodiazepine receptors found on platelets are thought to regulate stress at a cellular level [19]. However, in our studies flunitrazepam and, to a lesser extent, diazepam, inhibited aggregation, i.e. reversed the effects of GABA plus ionophore.

Wortmannin inhibits PI 3-kinase [11, 20] in a dose dependent manner [27]. PI 3-kinase acts as a focal point in two intracellular signal transduction pathways: the G-protein receptor ($\beta\ \gamma$ subunits) systems and the tyrosine kinase-linked (TKL) receptor systems [21]. Both pathways have been demonstrated in platelets. Although PI 3-kinase is considered to be a cytosolic enzyme, agonist activation can translocate it to the cytoskeleton where it is optimally positioned to influence plasma membrane and platelet shape [22]. Inhibition of PI 3-kinase by wortmannin reduces the availability of the second messenger phosphatidyl inositol 3,4,5-trisphosphate (PIP-3). The precise role of PIP-3 in platelets is unclear. However, in other intact cells, it appears at an early point in a pathway that leads to protein synthesis [21]. In addition, PIP-3 transduces many of the effects of insulin interaction with tyrosine kinase-linked (TKL) receptors [11].

The observations that verapamil and diltiazem, prototypical calcium channel blockers, were also effective inhibitors of GABA plus ionophore aggregation provide additional information about the importance of calcium influx for aggregation. Epinephrine and serotonin, when added in combination at subthreshold concentrations, synergistically evoke platelet aggregation [23]. Both amines activate G-protein coupled membrane receptors that raise the concentration of calcium via the IP_3 (inositol 1,4,5-trisphosphate) cascade. Wortmannin also inhibited monoamine-evoked aggregation [23], even though wortmannin has no effect on mobilization of calcium from the endoplasmic reticulum, or on influx of calcium [11]. Thus, the site of action of wortmannin must be at an aggregation effector independent of IP_3 and calcium, but dependent on PIP-3 produced by the enzymatic activity of PI 3-kinase.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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