

Adrenaline potentiates PI 3-kinase in platelets stimulated with thrombin and SFRLN: role of secreted ADP

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Abstract Adrenaline significantly potentiated late thrombin- and SFRLN-induced PtdIns(3,4)P₂ production. Furthermore, the potentiating effect of adrenaline on thrombin-induced PtdIns(3,4)P₂ production was independent on secreted ADP, whereas, the effect of adrenaline on SFRLN-induced PtdIns(3,4)P₂ production was completely dependent of secreted ADP. However, the ADP-dependent accumulation of PtdIns(3,4)P₂ was not required for irreversible platelet aggregation induced by SFRLN in the presence of adrenaline. It is concluded that adrenaline can replace secreted ADP to potentiate PtdIns(3,4)P₂ production in thrombin-stimulated but not in SFRLN-stimulated platelets, thus demonstrating a qualitative difference between platelet stimulation by thrombin and the thrombin receptor activating peptide SFRLN. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Adrenaline; Phosphoinositide 3-kinase

1. Introduction

Most agonists stimulate platelets by intracellular mobilization of Ca²⁺ through activation of poly phosphoinositide-specific phospholipase C (PLC) isozymes, although some agonist receptors are also coupled to channels facilitating influx of extracellular Ca²⁺ [1]. We have previously demonstrated that adrenaline powerfully potentiates agonist-induced platelet aggregation and dense granule secretion in an α_2 -adrenergic manner, whereas the catecholamine failed to stimulate these responses alone; the mechanism underlying the potentiating effects of adrenaline is not yet known but is regarded to be upstream of, or including, activation of PLC [2]. Over the last decade it has, however, become apparent that many agonists also activate phosphoinositide 3-kinase (PI3K) in platelets, by mechanisms not involving PLC or mobilization of Ca²⁺, and

that the 3-phosphorylated inositides have many functional targets in platelets [3].

We have recently demonstrated that the late production of 3-phosphorylated phosphoinositides in thrombin-stimulated platelets is dependent on synergistic interplay between individual signal transduction pathways initiated by autocrine agonists (ADP, thromboxane A₂, integrin ligation) formed by activated platelets [4]. Especially, ADP secreted from platelet dense granules plays a crucial role for thrombin- and SFRLN-induced late accumulation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂) [4,5]. In view of these results we explored in this communication if late thrombin- and SFRLN-stimulated PtdIns(3,4)P₂ production would be potentiated by exogenous added adrenaline. In the present study we show that adrenaline exerts a strong potentiating effect on thrombin- and SFRLN-induced synthesis of PtdIns(3,4)P₂ and that this potentiation differs completely between the two agonists with respect to requirement for secreted ADP.

2. Materials and methods

2.1. Materials

Thrombin was purchased from Parke-Davis, Morris Plains, NJ, USA. Prof. Nils Olav Solum (Rikshospitalet, Oslo, Norway) generously provided the synthetic thrombin receptor agonist peptide Ser-Phe-Arg-Leu-Leu-Asn (SFRLN). Thrombin and SFRLN were diluted with 0.15 M NaCl to desired concentrations just before the experiments. Adrenaline was obtained from Nycomed-Pharma, Oslo, Norway. Sepharose CL-2B was from Pharmacia Biotech, Sweden. ADP, CP (creatine phosphate), CPK (creatine phosphokinase), the PI 3-kinase inhibitor wortmannin and LY294002 were supplied by Sigma, St. Louis, MO, USA. RGDS (Arg-Gly-Asp-Ser, inhibitor of fibrinogen binding to integrin $\alpha_{IIb}\beta_3$) was obtained from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). [³²P]Orthophosphate ([³²P]P_i) was from Amersham International plc, UK.

2.2. Methods

2.2.1. Platelet isolation, labelling and incubation. Platelet-rich plasma (PRP) was prepared from fresh human venous blood anticoagulated with acid citrate dextrose (ACD) obtained at the blood bank, Haukeland Hospital, Bergen, Norway. To achieve high incorporation of [³²P]P_i in the phosphoinositide experiments, the platelets in PRP were sedimented by centrifugation at 1200×g for 10 min and then suspended in a calcium- and phosphate-free Tyrode's buffer adjusted to pH 6.7 with ACD before incubation with [³²P]P_i (0.5 mCi/ml) for 1 h at 37°C. The platelets were then transferred by gel filtration into a calcium- and phosphate-free Tyrode's buffer containing 5 mM glucose and 0.2% bovine serum albumin at pH 7.3. The concentration of the gel-filtered platelets (GFP), determined by a Coulter Counter, was adjusted to 3.5×10⁸ cells/ml with the gel filtration solution.

Aggregation of GFP was measured in a chronolog dual channel

Abbreviations: ACD, acid citrate dextrose; CP, creatine phosphate; CPK, creatine phosphokinase; GFP, gel-filtered platelets; GroPIIns, glycerophosphoinositols; HPLC, high performance liquid chromatography; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PRP, platelet-rich plasma; TLC, thin layer chromatography

aggregation module interfaced to a PC (Chrono-log Whole Blood-Lumi ionized calcium aggregometer, Havertown, PA, USA). GFP was always preincubated 2 min at 37°C in the absence or presence of antagonists before stimulation with agonist (thrombin or SFRLN and/or adrenaline). Unless otherwise indicated, adrenaline was added 10 s after thrombin or SFRLN.

2.2.2. Extraction and analysis of 3-phosphorylated glycerophosphoinositides [4]. In principle, aliquots of platelet incubation mixtures were mixed with methanol/chloroform/HCl and phase separated. The organic phase was concentrated and subjected to thin layer chromatography (TLC). The radioactive spots of PtdIns(3,4)P₂ and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) were isolated and deacylated and finally subjected to gradient high performance liquid chromatography (HPLC) according to Auger et al [6]. The radioactive peaks were detected directly in the eluate by Cerenkov scintillation. The output from the monitor was recorded online with a standard PC.

2.2.3. Statistics. Results are presented as mean ± S.E.M. The statistical significance was determined by paired sample Student's *t*-test.

3. Results

3.1. Synergistic stimulatory effect of adrenaline on thrombin- and SFRLN-induced synthesis of PtdIns(3,4)P₂

Platelets were stirred with thrombin (0.035 U/ml and 1 U/ml) with or without adrenaline for 5 min before analysis of radiolabelled phosphoinositides by TLC/HPLC. Adrenaline by itself (10 µg/ml) failed to induce late (5 min) production of PtdIns(3,4)P₂ (results not shown). However, as shown in Fig. 1A, when the same concentration of adrenaline was added 10 s after thrombin (0.035 U/ml), it significantly enhanced the thrombin-induced synthesis of PtdIns(3,4)P₂. The addition of wortmannin (100 nM) or LY294002 (25 µM), two unrelated inhibitors of PI 3-K, totally abolished thrombin- plus adrenaline-induced formation of PtdIns(3,4)P₂ (data not shown). The synergistic effect of adrenaline on thrombin-induced production of PtdIns(3,4)P₂ seen at 0.035 U/ml was not observed at a high concentration of thrombin (1 U/ml) (Fig. 1A). In agreement with the results obtained for thrombin (0.035 U/ml) above, adrenaline enhanced the late (5 min) SFRLN-induced accumulation of PtdIns(3,4)P₂ at 40 and 100 µM of SFRLN by 307 ± 97% (S.E.M.) and 422 ± 129%, respectively (Fig. 1B).

3.2. Effect of adrenaline on thrombin- and SFRLN-induced synthesis of PtdIns(3,4)P₂ in the presence of ADP scavengers and RGDS

As earlier reported [7,8], adrenaline synergistically enhances thrombin-induced inositol phospholipid metabolism ([³²P]PA,

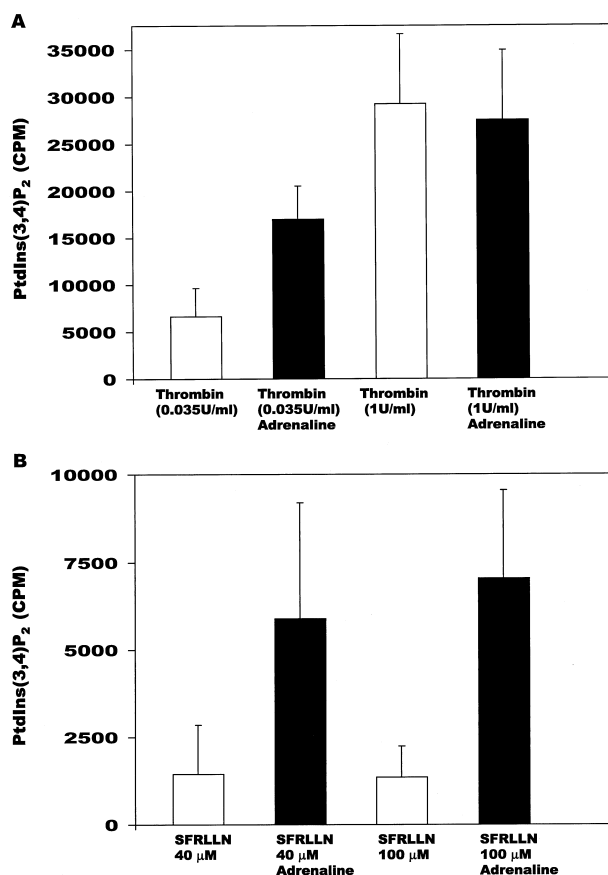


Fig. 1. Thrombin and SFRLN-induced accumulation of PtdIns(3,4)P₂ is potentiated by adrenaline. Platelets were stirred at 1000 rpm in a two-channel aggregometer for 5 min at 37°C with (A) thrombin (0.035 U/ml and 1 U/ml) or adrenaline plus thrombin together, or with (B) SFRLN (40 and 100 µM) or adrenaline (10 µg/ml) and SFRLN together. Adrenaline (10 µg/ml) was added 10 s after stimulation with thrombin or SFRLN. Radiolabelled deacylated glycerophosphoinositols (GroPIs) were analyzed by HPLC as described in Section 2. A: The data for late (5 min) thrombin-induced PtdIns(3,4)P₂ formation are the average of three different experiments, bars ± S.E.M. *P* < 0.05 for thrombin (0.035 U/ml)+vehicle vs. thrombin (0.035 U/ml)+adrenaline. B: The data for late (5 min) SFRLN-induced PtdIns(3,4)P₂ formation represent averages of three separate experiments performed in duplicate with 40 µM of SFRLN, and two separate experiments performed in duplicate with 100 µM of SFRLN, bars ± S.E.M. *P* < 0.05 for SFRLN (40 µM)+vehicle vs. SFRLN (40 µM)+adrenaline.

Table 1

Effect of adrenaline on thrombin-induced synthesis of PtdIns(3,4)P₂ in the presence of CP/CPK

Agonist	PtdIns(3,4)P ₂ (%)					
	Vehicle	Adrenaline	CP/CPK	CP/CPK+adrenaline	CP/CPK+RGDS	CP/CPK+RGDS+adrenaline
Thrombin (0.035 U/ml)	100 ± 45	267 ± 53 ^a	30 ± 11	189 ± 23 ^b	ND	ND
Thrombin (1 U/ml)	100 ± 10	96 ± 23	33 ± 15	79 ± 15 ^c	6 ± 3	17 ± 5 ^d

Platelets were preincubated 2 min at 37°C with or without CP/CPK (5 mM/10 U/ml) and RGDS (150 µM) before activation with thrombin (0.035 U/ml and 1 U/ml) or adrenaline (10 µg/ml) plus thrombin together for 5 min as described in Fig. 1. The amount of PtdIns(3,4)P₂ formation in the presence of thrombin alone was set at 100%. The data for late (5 min) PtdIns(3,4)P₂ formation are the average of three different experiments performed in duplicate, mean ± S.E.M.

^a*P* < 0.05 for thrombin (0.035 U/ml)+vehicle vs. thrombin (0.035 U/ml)+adrenaline.

^b*P* < 0.05 for thrombin (0.035 U/ml)+CP/CPK+vehicle vs. thrombin (0.035 U/ml)+CP/CPK+adrenaline.

^c*P* < 0.05 for thrombin (1 U/ml)+CP/CPK+vehicle vs. thrombin (1 U/ml)+CP/CPK+adrenaline.

^d*P* < 0.05 for thrombin (1 U/ml)+CP/CPK+RGDS+vehicle vs. thrombin (1 U/ml)+CP/CPK+RGDS+adrenaline.

[32 P]PIP $_2$ and [32 P]PIP) and secretion of ADP+ATP from dense granules at low concentrations of thrombin. Since secreted ADP plays a key role for thrombin- and SFRLN-induced late accumulation of PtdIns(3,4)P $_2$ [4,5], we next investigated whether adrenaline functions as a synergistic potentiator on PtdIns(3,4)P $_2$ synthesis also in the presence of the ADP scavenger system CP/CPK.

The presence of CP/CPK almost abolished the formation of PtdIns(3,4)P $_2$ at 0.035 U/ml of thrombin alone, whereas the synergistic effect of adrenaline on thrombin-induced (0.035 U/ml) PtdIns(3,4)P $_2$ synthesis only was slightly reduced (Table 1 and Fig. 1A). Furthermore, when secreted ADP was removed by CP/CPK we observed synergism between adrenaline and thrombin on the formation of PtdIns(3,4)P $_2$ also at higher concentrations of thrombin (1 U/ml) (Table 1), in contrast to the lack of effect of adrenaline when secreted ADP was allowed to be present (Fig. 1A). The synergistic effect of adrenaline on thrombin-induced PtdIns(3,4)P $_2$ synthesis was also found in the presence of both CP/CPK and RGDS which inhibits fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (Table 1).

In sharp contrast, the ADP removing enzyme system CP/CPK eliminated the late SFRLN-induced (100 μ M) production of PtdIns(3,4)P $_2$ both in the presence and absence of adrenaline (Fig. 2).

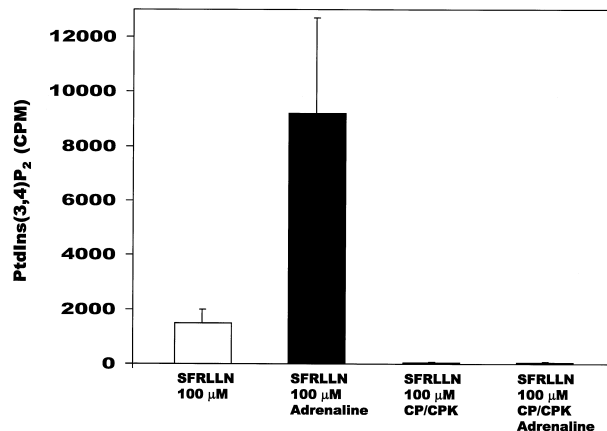


Fig. 2. Effect of adrenaline on SFRLN-induced synthesis of PtdIns(3,4)P $_2$ in the presence of CP/CPK. Platelets were preincubated 2 min at 37°C with or without CP/CPK before activation with SFRLN (100 μ M) or adrenaline (10 μ g/ml) and SFRLN together. The measurement of SFRLN-induced PtdIns(3,4)P $_2$ formation was done after 5 min as described in Fig. 1. The data are representative of two separate experiments performed in duplicate, bars $\pm$ S.E.M.

Optical aggregation

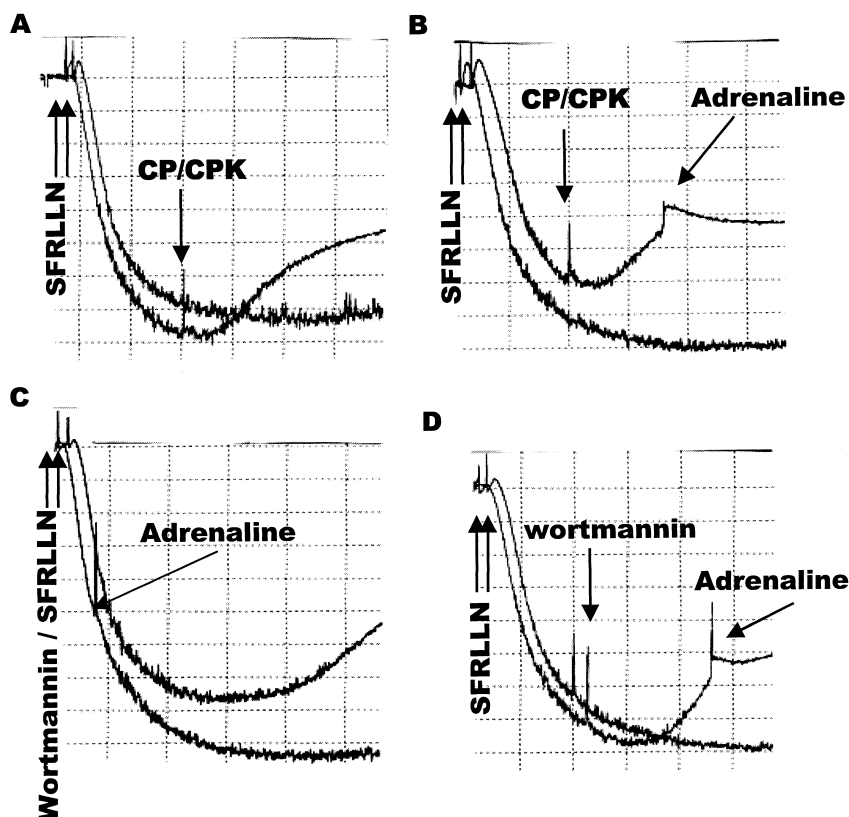


Fig. 3. Inhibition of irreversible aggregation by the PI 3-K inhibitor wortmannin or the ADP-scavenger system CP/CPK is restored by adrenaline. GFP were stirred at 1000 rpm measured in a chronolog dual channel aggregation module interfaced to a PC. Arrows show additions. A: SFRLN (40 μ M)+vehicle versus SFRLN (40 μ M)+CP/CPK (5 mM/10 U/ml). B: SFRLN (40 μ M)+vehicle+vehicle versus SFRLN (40 μ M)+CP/CPK (5 mM/10 U/ml)+adrenaline (10 μ g/ml). C: Platelets were preincubated 2 min at 37°C with 100 nM wortmannin before activation with SFRLN (40 μ M)+vehicle or SFRLN (40 μ M)+adrenaline (10 μ g/ml). D: SFRLN (40 μ M)+vehicle+vehicle vs. SFRLN (40 μ M)+wortmannin (100 nM)+adrenaline (10 μ g/ml).

3.3. Inhibition of irreversible aggregation by the PI 3-K inhibitor wortmannin is restored by adrenaline

Fig. 3 shows SFRLN (40 μ M)-induced aggregation in the presence and absence of CP/CPK (5 mM/10 U/ml), wortmannin (100 nM) and adrenaline (10 μ g/ml).

Addition of CP/CPK, which removes secreted ADP, reversed SFRLN-induced platelet aggregation (Fig. 3A), as previously shown by using the ADPase enzyme apyrase [9]. The addition of adrenaline 90 s after the CP/CPK addition restored the SFRLN-induced irreversible aggregation (Fig. 3B).

As shown in Fig. 2, PtdIns(3,4)P₂ production in platelets coactivated with SFRLN and adrenaline was completely abolished when platelets were preincubated with CP/CPK. Thus, potentiation of SFRLN-induced platelet aggregation by adrenaline seems to be independent of the 3-phosphorylated phosphoinositide PtdIns(3,4)P₂. Consistent with this hypothesis, Fig. 3C shows that reversion of SFRLN-induced irreversible aggregation by the PI 3-K inhibitor wortmannin (2 min preincubation) was prevented by addition of adrenaline. As recently reported [5], addition of wortmannin 2 min after SFRLN-stimulation reversed platelet aggregation (Fig. 3D). However, addition of adrenaline 2 min after the wortmannin addition recovered the SFRLN-induced platelet aggregation (Fig. 3D).

4. Discussion

In the present study we demonstrated that adrenaline has a strong synergistic potentiating effect on thrombin- and SFRLN-induced synthesis of PtdIns(3,4)P₂. The involvement of secreted ADP in this potentiation differed very much between the two agonists, as the potentiation of SFRLN-induced PtdIns(3,4)P₂ production was abolished by the presence of CP/CPK, while for thrombin it was actually further potentiated by CP/CPK. Particularly, at the high concentration (1.0 U/ml) of thrombin adrenaline did not potentiate PtdIns(3,4)P₂ production when secreted ADP was allowed to be present, while adrenaline caused a strong potentiation when secreted ADP was removed. One plausible explanation could be that when secreted ADP accumulated extracellularly, it potentiated PtdIns(3,4)P₂ production and the PtdIns(3,4)P₂ production seen was the result of both thrombin and ADP action on the platelets. When ADP was removed by CP/CPK, only thrombin's action alone is measured which is then potentiated by adrenaline to the same extent as secreted ADP. The potentiation by adrenaline of SFRLN-induced PtdIns(3,4)P₂ production obviously depends on secreted ADP in another way than thrombin. Previous work has showed that SFRLN-induced platelet activation is less potent, and acts through different signalling pathways, than thrombin-induced platelet activation [9]. Thrombin cleaves the Arg⁴¹–Ser⁴² peptide bond of the G-protein-coupled, protease-activated receptor PAR1 in human platelets, thereby releasing a 41 amino acid peptide (TR_{1–41}) and producing a new amino-terminus starting with the sequence SFRLN that acts as a tethered peptide ligand [10–12]. Interestingly, TR_{1–41} is a strong platelet agonist, which is almost as potent as thrombin [13]. Moreover, studies with wortmannin indicated that TR_{1–41}-induced downstream signalling pathways involve PI3K [13]. We found that the synergistic effect of adrenaline on SFRLN-induced PtdIns(3,4)P₂

production was completely dependent on secreted ADP. Platelets coactivated with ADP and adrenaline did not induce synthesis of PtdIns(3,4)P₂ (data not shown). However, as discussed above, the potentiating effect of adrenaline on thrombin-induced PtdIns(3,4)P₂ production seemed to replace the potentiating effect of secreted ADP. One major difference between the physiological tethered ligand produced by thrombin and the added peptide ligand fragment SFRLN is that adrenaline can not replace secreted ADP in the case of SFRLN, while it seems to replace ADP in the case of thrombin. Synergism between adrenaline and thrombin on the formation of PtdIns(3,4)P₂ was also found in the presence of both CP/CPK and RGDS that block fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (Table 1). In light of the previous discussion we therefore speculate that crosstalk between signalling transduction pathways initiated by adrenaline and TR_{1–41} has a synergistic stimulatory effect on PI3K in platelets. Furthermore, in preliminary experiments we found that the α_2 -adrennergic antagonist yohimbine totally abolished the potentiating effect of adrenaline on thrombin-induced PA formation and PtdIns(3,4)P₂ production (data not shown). Thus, the synergistic stimulatory effect of adrenaline on thrombin-induced PA formation and PtdIns(3,4)P₂ production seems to be transduced through α_2 -adrenoreceptors.

By using the ADP scavenger CP/CPK- and the PI3K inhibitor wortmannin, we confirmed previous work [5,9,14] showing that ADP and the PI3K product PtdIns(3,4)P₂ are necessary for SFRLN (PAR1)-induced irreversible platelet aggregation (Figs. 2 and 3). However, we found further that addition of adrenaline recovered the CP/CPK- and wortmannin-blocked SFRLN-induced platelet aggregation (Fig. 3). Thus, ADP signalling and late accumulation of the PI3K product PtdIns(3,4)P₂ is not required for irreversible platelet aggregation induced by SFRLN in the presence of adrenaline, while it is apparently necessary for PtdIns(3,4)P₂ production. Interestingly, the potentiating effect of adrenaline of agonist-induced platelet aggregation and associated inhibition of cAMP formation were recently reported to be strongly inhibited or absent in knockout mice which lack the α subunit of the heterotrimeric G protein G_z (G_z α (–/–)) [15]. Thus, the α_2 -adrenergic downstream effector G_z α seems to play a crucial role in mediating irreversible aggregation in platelets.

In summary, we demonstrate a strong synergistic effect of adrenaline on thrombin- and SFRLN-induced PtdIns(3,4)P₂ production. The synergism between adrenaline and thrombin on the accumulation of PtdIns(3,4)P₂ is independent on secreted ADP, whereas the synergistic effect of adrenaline on SFRLN-induced PtdIns(3,4)P₂ synthesis is entirely dependent on secreted ADP. However, ADP-dependent late synthesis of PtdIns(3,4)P₂ is not required for SFRLN-induced irreversible platelet aggregation in the presence of adrenaline.

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