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

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Abstract Adrenaline significantly potentiated late thrombin- and SFRLLN-induced PtdIns(3,4)P_2 production. Furthermore, the potentiating effect of adrenaline on thrombin-induced PtdIns(3,4)P_2 production was independent on secreted ADP, whereas, the effect of adrenaline on SFRLLN-induced PtdIns(3,4)P_2 production was completely dependent of secreted ADP. However, the ADP-dependent accumulation of PtdIns(3,4)P_2 was not required for irreversible platelet aggregation induced by SFRLLN in the presence of adrenaline. It is concluded that adrenaline can replace secreted ADP to potentiate PtdIns(3,4)P_2 production in thrombin-stimulated but not in SFRLLN-stimulated platelets, thus demonstrating a qualitative difference between platelet stimulation by thrombin and the thrombin receptor activating peptide SFRLLN.

Key words: Adrenaline; Phosphoinositide 3-kinase

1. Introduction

Most agonists stimulate platelets by intracellular mobilization of Ca^{2+} through activation of poly phosphoinositide-specific phospholipase C (PLC) isozymes, although some agonist receptors are also coupled to channels facilitating influx of extracellular Ca^{2+} [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 Ca^{2+}, 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 A_2, integrin ligation) formed by activated platelets [4]. Especially, ADP secreted from platelet dense granules plays a crucial role for thrombin- and SFRLLN-induced late accumulation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) [4,5]. In view of these results we explored in this communication if late thrombin- and SFRLLN-stimulated PtdIns(3,4)P_2 production would be potentiated by exogenous added adrenaline. In the present study we show that adrenaline exerts a strong potentiating effect on thrombin- and SFRLLN-induced synthesis of PtdIns(3,4)P_2 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 (SFRLLN). Thrombin and SFRLLN 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). [^{32}P]Orthophosphate ([^{32}P]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 [^{32}P]P_i in the phosphoinositide experiments, the platelets in PRP were sedimented by centrifugation at $1200\times g$ 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 [^{32}P]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 3.5×10^8 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; GroPIns, glycerophosphoinositols; HPLC, high performance liquid chromatography; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PRP, platelet-rich plasma; TLC, thin layer chromatography

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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 SFRLLN and/or adrenaline). Unless otherwise indicated, adrenaline was added 10 s after thrombin or SFRLLN.

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)P_2 and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) 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 SFRLLN-induced synthesis of PtdIns(3,4)P_2

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)P_2 (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)P_2. 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)P_2 (data not shown). The synergistic effect of adrenaline on thrombin-induced production of PtdIns(3,4)P_2 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) SFRLLN-induced accumulation of PtdIns(3,4)P_2 at 40 and 100 μM of SFRLLN by 307 ± 97% (S.E.M.) and 422 ± 129%, respectively (Fig. 1B).

3.2. Effect of adrenaline on thrombin- and SFRLLN-induced synthesis of PtdIns(3,4)P_2 in the presence of ADP scavengers and RGDS

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

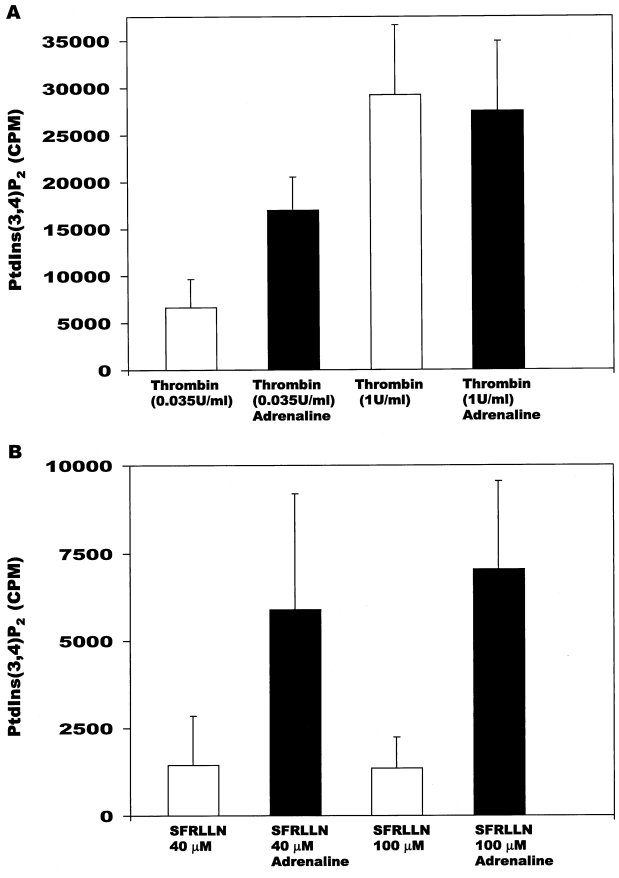

Fig. 1. Thrombin and SFRLLN-induced accumulation of PtdIns(3,4)P_2 is potentiated by adrenaline. Platelets were stirred at 1000 rpm in a two-channel aggregometer for 5 min at 37°C with (A) thrombin (0.035 U/ml and 1 U/ml) or adrenaline plus thrombin together, or with (B) SFRLLN (40 and 100 μM) or adrenaline (10 μg/ml) and SFRLLN together. Adrenaline (10 μg/ml) was added 10 s after stimulation with thrombin or SFRLLN. Radiolabelled deacylated glycerophosphoinositols (GroPIns) were analyzed by HPLC as described in Section 2. A: The data for late (5 min) thrombin-induced PtdIns(3,4)P_2 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) SFRLLN-induced PtdIns(3,4)P_2 formation represent averages of three separate experiments performed in duplicate with 40 μM of SFRLLN, and two separate experiments performed in duplicate with 100 μM of SFRLLN, bars ± S.E.M. $P < 0.05$ for SFRLLN (40 μM)+vehicle vs. SFRLLN (40 μM)+adrenaline.

Table 1
Effect of adrenaline on thrombin-induced synthesis of PtdIns(3,4)P_2 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)P_2 formation in the presence of thrombin alone was set at 100%. The data for late (5 min) PtdIns(3,4)P_2 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 SFRLLN-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 α_{IIb}/β_3 (Table 1).

In sharp contrast, the ADP removing enzyme system CP/CPK eliminated the late (5 min) SFRLLN-induced (100 μM) production of PtdIns(3,4)P_2 both in the presence and absence of adrenaline (Fig. 2).

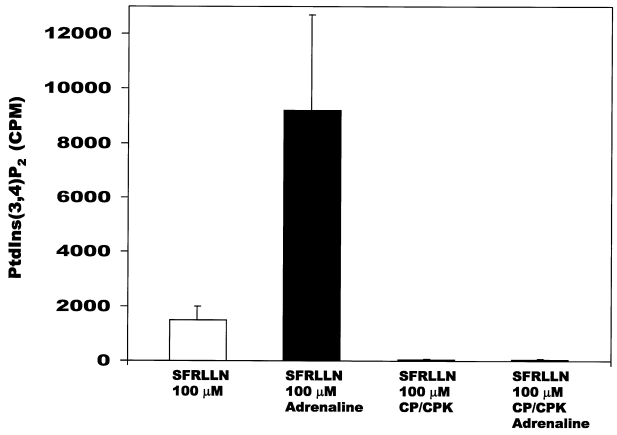

Fig. 2. Effect of adrenaline on SFRLLN-induced synthesis of PtdIns(3,4)P_2 in the presence of CP/CPK. Platelets were preincubated 2 min at 37°C with or without CP/CPK before activation with SFRLLN (100 μM) or adrenaline (10 μg/ml) and SFRLLN together. The measurement of SFRLLN-induced PtdIns(3,4)P_2 formation was done after 5 min as described in Fig. 1. The data are representative of two separate experiments performed in duplicate, bars ± S.E.M.

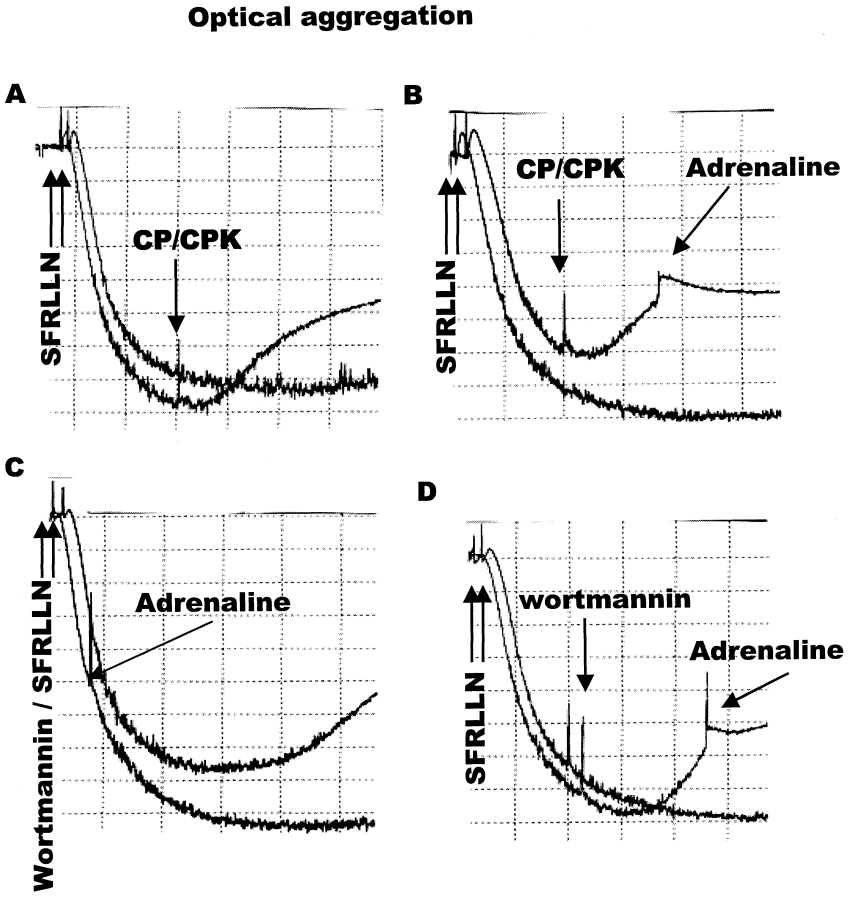

Fig. 3. Inhibition of irreversible aggregation by the PI 3-K inhibitor wortmannin or the ADP-scavenger system CP/CPK is restored by adrenaline. GFP were stirred at 1000 rpm measured in a chronolog dual channel aggregation module interfaced to a PC. Arrows show additions. A: SFRLLN (40 μM)+vehicle versus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

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

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

Addition of CP/CPK, which removes secreted ADP, reversed SFRLLN-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 SFRLLN-induced irreversible aggregation (Fig. 3B).

As shown in Fig. 2, PtdIns(3,4)P_2 production in platelets coactivated with SFRLLN and adrenaline was completely abolished when platelets were preincubated with CP/CPK. Thus, potentiation of SFRLLN-induced platelet aggregation by adrenaline seems to be independent of the 3-phosphorylated phosphoinositide PtdIns(3,4)P_2. Consistent with this hypothesis, Fig. 3C shows that reversion of SFRLLN-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 SFRLLN-stimulation reversed platelet aggregation (Fig. 3D). However, addition of adrenaline 2 min after the wortmannin addition recovered the SFRLLN-induced platelet aggregation (Fig. 3D).

4. Discussion

In the present study we demonstrated that adrenaline has a strong synergistic potentiating effect on thrombin- and SFRLLN-induced synthesis of PtdIns(3,4)P_2. The involvement of secreted ADP in this potentiation differed very much between the two agonists, as the potentiation of SFRLLN-induced PtdIns(3,4)P_2 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)P_2 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)P_2 production and the PtdIns(3,4)P_2 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 SFRLLN-induced PtdIns(3,4)P_2 production obviously depends on secreted ADP in another way than thrombin. Previous work has showed that SFRLLN-induced platelet activation is less potent, and acts through different signalling pathways, than thrombin-induced platelet activation [9]. Thrombin cleaves the Arg^{41}–Ser^{42} 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 SFRLLN 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 SFRLLN-induced PtdIns(3,4)P_2 production was completely dependent on secreted ADP. Platelets coactivated with ADP and adrenaline did not induce synthesis of PtdIns(3,4)P_2 (data not shown). However, as discussed above, the potentiating effect of adrenaline on thrombin-induced PtdIns(3,4)P_2 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 SFRLLN is that adrenaline can not replace secreted ADP in the case of SFRLLN, while it seems to replace ADP in the case of thrombin. Synergism between adrenaline and thrombin on the formation of PtdIns(3,4)P_2 was also found in the presence of both CP/CPK and RGDS that block fibrinogen binding to integrin α_{IIb}/β_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-adrenergic antagonist yohimbine totally abolished the potentiating effect of adrenaline on thrombin-induced PA formation and PtdIns(3,4)P_2 production (data not shown). Thus, the synergistic stimulatory effect of adrenaline on thrombin-induced PA formation and PtdIns(3,4)P_2 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)P_2 are necessary for SFRLLN (PAR1)-induced irreversible platelet aggregation (Figs. 2 and 3). However, we found further that addition of adrenaline recovered the CP/CPK- and wortmannin-blocked SFRLLN-induced platelet aggregation (Fig. 3). Thus, ADP signalling and late accumulation of the PI3K product PtdIns(3,4)P_2 is not required for irreversible platelet aggregation induced by SFRLLN in the presence of adrenaline, while it is apparently necessary for PtdIns(3,4)P_2 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 ($G_{z\alpha}$ (−/−))[15]. Thus, the α_2-adrenergic downstream effector $G_{z\alpha}$ 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 SFRLLN-induced PtdIns(3,4)P_2 production. The synergism between adrenaline and thrombin on the accumulation of PtdIns(3,4)P_2 is independent on secreted ADP, whereas the synergistic effect of adrenaline on SFRLLN-induced PtdIns(3,4)P_2 synthesis is entirely dependent on secreted ADP. However, ADP-dependent late synthesis of PtdIns(3,4)P_2 is not required for SFRLLN-induced irreversible platelet aggregation in the presence of adrenaline.

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