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ADP, adrenaline and serotonin stimulate inositol 1,4,5-trisphosphate production in human platelets

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Abstract

Although adenosine diphosphate (ADP) is a well-known stimulus of platelet aggregation, it is not the generally accepted view that ADP stimulates phosphatidylinositolbisphosphate (PtdIns(4,5)P_2) hydrolysis. Using a very sensitive competitive receptor binding assay for inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), we have detected Ins(1,4,5)P_3 production at early (< 10 s) time points after stimulation of human platelets by the weak agonists ADP, adrenaline and serotonin (5-hydroxytryptamine, 5-HT). When adrenaline or 5-HT was combined with ADP in the presence of aspirin, there was a significant potentiation of ADP-induced platelet aggregation, but there was no potentiation of Ins(1,4,5)P_3 generation. Also, the increases in intracellular calcium (Ca^{2+}) concentrations stimulated by ADP were not potentiated by adrenaline in the presence of aspirin. Therefore, the synergism between the purinergic and adrenergic pathways of platelet activation occurs downstream from PtdIns(4,5)P_2 hydrolysis and intracellular Ca^{2+} mobilization, although prior to platelet aggregation.

Keywords: ADP; 5-HT (5-hydroxytrytamine, serotonin); Adrenaline; Inositol 1,4,5-trisphosphate; Human platelet; Synergism; Ca^{2+}

1. Introduction

Although adenosine diphosphate (ADP) and adrenaline are well-known stimuli of platelet aggregation, there is controversy over their ability to stimulate phosphatidylinositolbisphosphate (PtdIns(4,5)P_2) hydrolysis. (Lloyd et al., 1972, 1973a,b; Vickers et al., 1982, 1986, 1990; Siess et al., 1984; Fisher et al., 1985; MacIntyre et al., 1985; Daniel et al., 1986, 1987; Duncan et al., 1992; Blockmans et al., 1995). There is considerable evidence that the hydrolysis of PtdIns(4,5)P_2 and the formation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) is an initial and key step in the triggering of the platelet aggregation response by strong agonists such as thrombin and collagen (Lloyd and Mustard, 1974; Agranoff et al., 1983; Rittenhouse and Sasson, 1985; Daniel et al., 1987; Tysnes et al., 1991). The lack of convincing evidence for ADP-induced PtdIns(4,5)P_2 hydrolysis has led to the search for other mechanisms of action for ADP (MacIntyre et al., 1985; Sage and Rink, 1987). A very sensitive receptor binding assay for Ins(1,4,5)P_3 has provided a significant advance in the study of this mediator in other cells (Challiss et al., 1988; Bredt et al., 1989) and offers an opportunity to reexamine the signal transduction mechanisms of platelet activation by the weak agonists ADP, adrenaline and serotonin (5-hydroxytryptamine, 5-HT).

We have previously shown that the synergistic platelet aggregation response induced by combinations of ADP and adrenaline or 5-HT is a specific receptor-mediated process inhibited by the specific receptor antagonists yohimbine and ketanserin and not entirely dependent on the effects of thromboxane (Vanags et al., 1992). In the present study we have examined whether the synergistic platelet aggregation response by these weak agonists is mediated by synergism at the level of signal transduction, that is, by a potentiation of Ins(1,4,5)P_3 formation and intracellular calcium (Ca^{2+}) mobilisation.

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2. Materials and methods

2.1. Materials

[^{3}H]myo-inositol-1,4,5-trisphosphate (specific activity 17–21 Cimmol^{-1}) was from New England Nuclear Research Products (Boston, MA, USA). D-myo-inositol 1,4,5-trisphosphate (potassium salt) was from Amersham International (Little Chalfont, UK). ADP, 5-HT creatinine sulphate complex, Hepes, dimethyl sulphoxide and albumin fraction V were obtained from Sigma (St. Louis, MO, USA). Adrenaline tartrate was from Astra Pharmaceuticals. Aequorin (lyophilized) was from Dr. Blinks (Mayo Clinics, Rochester, MN, USA). Acetyl-salicylic acid (aspirin) was obtained from BDH. Apyrase was prepared from potatoes according to the method of Molner and Lorand (1961). All other compounds were of analytical grade.

2.2. Preparation of platelet suspensions for aggregation and Ins(1,4,5)P_3 experiments

Venous blood was taken by venepuncture from healthy volunteers into 6/1 (v/v) acid-citrate-dextrose (pH 4.5). Washed platelet suspensions were prepared according to the method of Mustard et al. (1989). Washing buffer contained heparin (50 IU/ml), apyrase (7 μl/ml) and $CaCl_2$ (2 mM), (pH adjusted to 6.5) and the resuspension buffer contained $CaCl_2$ (1 mM) (pH 7.4). The platelet suspension was stored in a stoppered air-free syringe (to prevent the loss of CO_2 and changes in pH). Platelet counts were adjusted to 300 000/μl for tests of aggregation and to 600 000/μl for experiments on Ins(1,4,5)P_3 production. Platelets were incubated at 37°C for 30 min in the presence of aspirin (100 μM) in all experiments in which Ins(1,4,5)P_3 was measured.

2.3. Platelet aggregation

Platelet aggregation and the preceding shape change of the washed platelets in response to ADP were measured by changes in light transmission in an aggregometer (Payton, Ontario, Canada). Platelet-free plasma was added (1.5 μl/300 μl platelet suspension) as a source of fibrinogen.

2.4. Ins(1,4,5)P_3 production

Addition of platelet-free plasma (5 μl) to the washed platelet suspension (895 μl), stirred at 600 rpm in a siliconised glass cuvette, was followed immediately by the simultaneous addition of the two stimuli or buffer (50 μl each). After stimulation, each platelet sample was extracted with ice-cold perchloric acid (100 μl, 6.6 M) and placed on ice for 20 min. The protein precipitate was sedimented by centrifugation for 15 min at 4°C and 2000 $\times g$. The supernatant was neutralized by careful titration with KOH in the presence of Universal Indicator (20 μl). The potassium perchlorate salt was removed by centrifugation for 15 min at 4°C and 2000 $\times g$. The supernatants were evaporated to dryness under vacuum and reconsti-

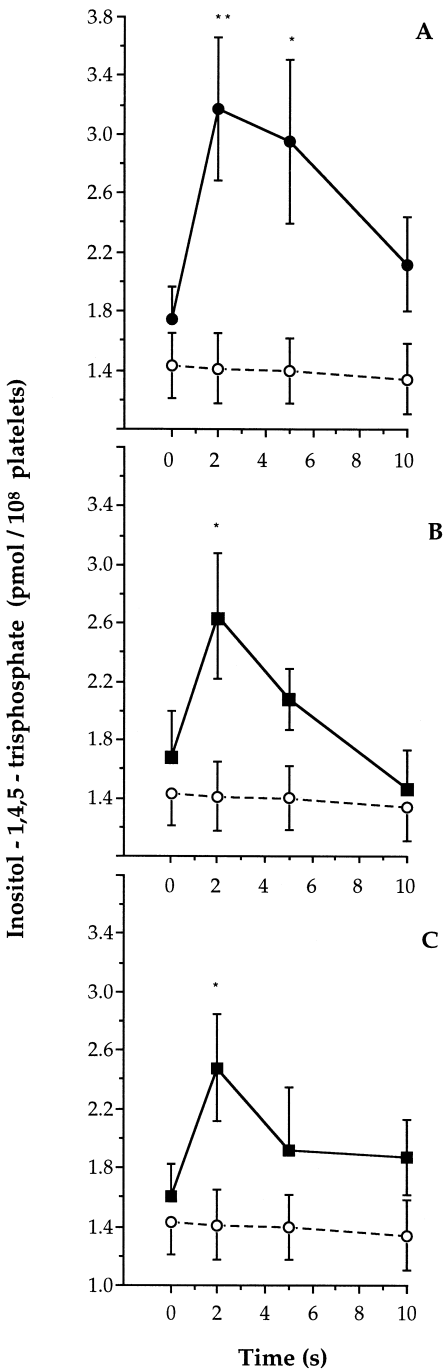

Fig. 1. The time-course of Ins(1,4,5)P_3 formation in washed human platelets stimulated by the weak agonists in the presence of aspirin (A) ADP 500 μM (filled circles) (B) adrenaline 10 μM (filled squares) (C) 5-HT 10 μM (filled squares) or buffer control (open circles). The experiments were performed in the presence of aspirin (100 μM) and platelet-free plasma (5 μl/ml as a fibrinogen source). Data are the mean ± sem ($n = 11$, with duplicate determinations in a randomised block design with respect to stimulus and time). $**$ ($P < 0.01$) and $*$ ($P < 0.05$) indicate a significant increase in Ins(1,4,5)P_3 production compared to buffer control at respective time points.

tuted in 250 μl distilled water. Reconstituted samples were stored at −80°C for several weeks without deterioration in the concentration of Ins(1,4,5)P_3. Platelet resuspension buffer was also extracted in the same manner and used as the diluent for the Ins(1,4,5)P_3 standards. The samples and standards were assayed in duplicate for Ins(1,4,5)P_3 concentration using the following adaptations to the method described by Bredt et al. (1989).

The platelet sample (100 μl) was combined with [^{3}H]Ins(1,4,5)P_3 (0.25 nM, specific activity 17–21 Ci/mmol) in the presence of Tris buffer (100 mM) plus mercaptoethanol (1 mM) and EDTA (4.5 mM; pH 8.4) in an incubation volume of 450 μl. Rat cerebellar membrane homogenate (50 μl, 0.12 mg of protein) was added and the samples incubated on ice for 10 min. Incubated identically were Ins(1,4,5)P_3 standards (0.19–200 pmol) which had been diluted in acid-extracted platelet resuspension buffer. The incubations were stopped by centrifugation for 10 min at 4°C and 2000 × g, the supernatants were removed, the pellet solubilised overnight and the radioactivity of the pellet determined. In this assay total binding ([^{3}H]Ins(1,4,5)P_3 in the absence of Ins(1,4,5)P_3) was routinely between 1500–2000 dpm and non-specific binding ([^{3}H]Ins(1,4,5)P_3 binding not displaced by 10 μM D-Ins(1,4,5)P_3), was approximately 6%. The counting efficiency in typical samples was 50%. The mean extraction efficiency was determined as 36% by the assay of internal standards in each experiment. The amount of Ins(1,4,5)P_3 in each sample was calculated using the standard curve, standardised for extraction efficiency and platelet count and finally expressed as pmol/10^8 platelets. The detection limit of the assay was 0.40 ± 0.02 pmol/10^8 platelets. Ins(1,4,5)P_3 receptors were prepared according to the method of Bredt et al. (1989). The cerebella of two rats provided enough binding protein for the assay of 37 samples and a 12 point standard curve which were both assayed in duplicate. The protein concentration of the cerebellar membrane receptor homogenate was measured using the Coomassie Blue technique (Bradford, 1976).

2.5. Calcium mobilisation

All donors denied taking medication for at least two weeks before blood collection. The platelet washing and aequorin loading method was that described by Potevin et al. (1990). The platelets were then suspended in HEPES suspension buffer (5 ml) containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM), at 3×10^5 platelets/μl. The platelet suspension was incubated at 37°C in a plastic syringe with air excluded. Platelet aggregation and luminescence were quantitated in the same aequorin-loaded platelet sample in a Platelet Ionized Calcium Aggregometer (PICA: Chronolog, Havertown, PA) at 37°C within 60–90 min of aequorin loading. Intracellular Ca^{2+} concentration was determined from the stimulated aequorin luminescence peak (L) using calibration curves of log fractional luminescence (L/L_{max}) vs. log Ca^{2+} concentration supplied with each batch of aequorin. Platelet suspensions which caused Ca^{2+} mobilisation but did not change shape and aggregate in response to ADP were discarded. Electron microscopy studies showed that platelet suspensions were not substantially activated after aequorin loading.

2.6. Statistical analysis

The data were analysed by the Friedman's nonparametric two-way analysis of variance by ranks followed by a multiple-comparison procedure.

2.7. Ethics approval

Collection of blood from human volunteers was approved by the Ethics Committee of the Royal Adelaide Hospital. Use of rats for measurement of Ins(1,4,5)P_3 concentrations was approved by the Ethics Committee of the University of Adelaide.

3. Results

3.1. Ins(1,4,5)P_3 production by the weak agonists

ADP (500 μM) stimulated an increase in the mass of Ins(1,4,5)P_3 at 2 s (Fig. 1A), which by 10 s had decreased

Table 1
The concentration–response relationship for Ins(1,4,5)P_3 formation at two seconds, induced by combinations of weak agonists, in the presence of aspirin

	Ins-(1,4,5 P)$_3$ (pmol/10^8 platelets) stimulated by				
	0 μM ADP	10 μM ADP	50 μM ADP	100 μM ADP	500 μM ADP
ADP alone	1.30 ± 0.28[a]	1.94 ± 0.27	2.40 ± 0.33[c]	1.92 ± 0.27	2.54 ± 0.20[c]
ADP + adrenaline	2.35 ± 0.38[b]	2.69 ± 0.30[c]	2.50 ± 0.33[c]	2.74 ± 0.37[c]	3.46 ± 0.52[c]
ADP + 5-HT	2.75 ± 0.38[c]	2.24 ± 0.31[b]	2.44 ± 0.41[b]	2.52 ± 0.35[c]	3.08 ± 0.30[c]

Platelet suspensions were stimulated for 2 s by the agonists alone or in combination (added simultaneously) and the amount of Ins(1,4,5)P_3 determined. The concentration of adrenaline was 1 μM and of 5-HT was 10 μM. The experiments were performed in the presence of aspirin (100 μM) and platelet-free plasma (5 μl/ml as a fibrinogen source). The data are the mean ± standard error of the mean (S.E.M.) of 7 separate experiments which were randomised with respect to stimulus and ADP concentration. A significant increase in the amount of Ins(1,4,5)P_3 compared to basal levels[a] is indicated by [b]($P < 0.05$) and [c]($P < 0.01$). A difference of 0.89 between means is statistically significant at the 0.05 level.

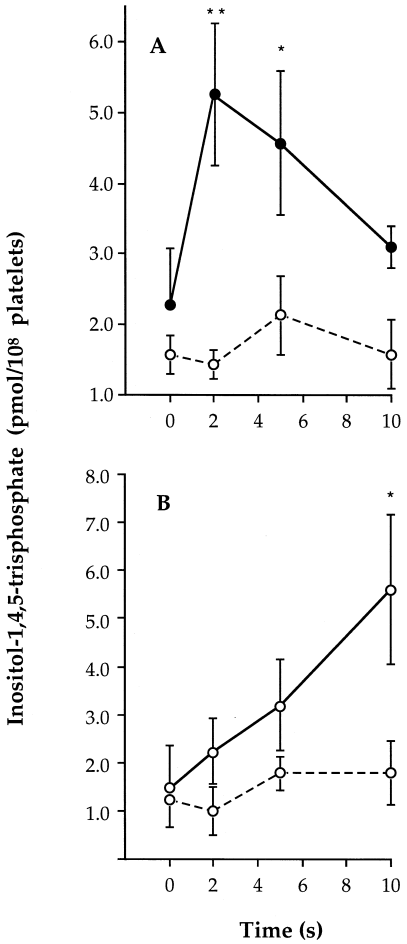

Fig. 2. The time-course of Ins(1,4,5)P_3 formation in washed human platelets stimulated by the strong agonists in the presence of aspirin. (A) Thrombin 1.5 IU/ml (filled circles) or buffer control (open circles) or (B) collagen 10 μg/ml (open circles, solid line) or buffer control (open circles, dashed line). The experiments were performed in the presence of aspirin (100 μM) and platelet-free plasma (5 μl/ml). Data are the mean ± sem ($n = 3$, with duplicate determinations for thrombin, $n = 4$, with duplicate determinations for collagen, in a randomised block design with respect to stimulus and time). $**(P < 0.01)$ and $*(P < 0.05)$ indicate a significant increase in Ins(1,4,5)P_3 production compared to buffer control at respective time points.

to levels not significantly above control. The production of Ins(1,4,5)P_3 by ADP followed a similar time course to the shape change response which occurs prior to aggregation (data not shown). The concentration–response relationship for ADP-induced Ins(1,4,5)P_3 generation showed that lower concentrations of ADP also stimulated increases in Ins(1,4,5)P_3 generation (Table 1). Adrenaline (10 μM) caused a smaller more transient increase in the mass of Ins(1,4,5)P_3 at 2 s (Fig. 1B) which by 5 s was slightly but not significantly above control. Production of Ins(1,4,5)P_3 induced by 5-HT (10 μM) was similar to that for adrenaline (Fig. 1C). For comparison, Ins(1,4,5)P_3 production induced by thrombin (1.5 IU/ml) and collagen (10 μg/ml) were also determined. Ins(1,4,5)P_3 production by thrombin was greatest at 2 s, as for the weak agonists (Fig. 2A), but for collagen a more delayed response was seen with an increase at 10 s after the addition of stimulus (Fig. 2B).

3.2. Synergistic platelet aggregation

Aggregation in response to increasing concentrations of ADP (1–500 μM) reached a plateau with 100 μM ADP (Fig. 3A. EC_{50} 12.5 μM, where EC_{50} is the effective concentration giving 50% maximum response). Adrenaline (10 μM) alone caused no platelet aggregation, but potentiated ADP-induced platelet aggregation as shown by the leftward displacement of the concentration response curve (EC_{50} 2.6 μM; a 4.8 fold decrease) and an increase in maximum response (Fig. 3A). 5-HT (10 μM) also caused a decrease in EC_{50} for ADP ($EC_{50} = 3$ μM, 4.2 fold decrease) and an increase in maximum response (Fig. 3B).

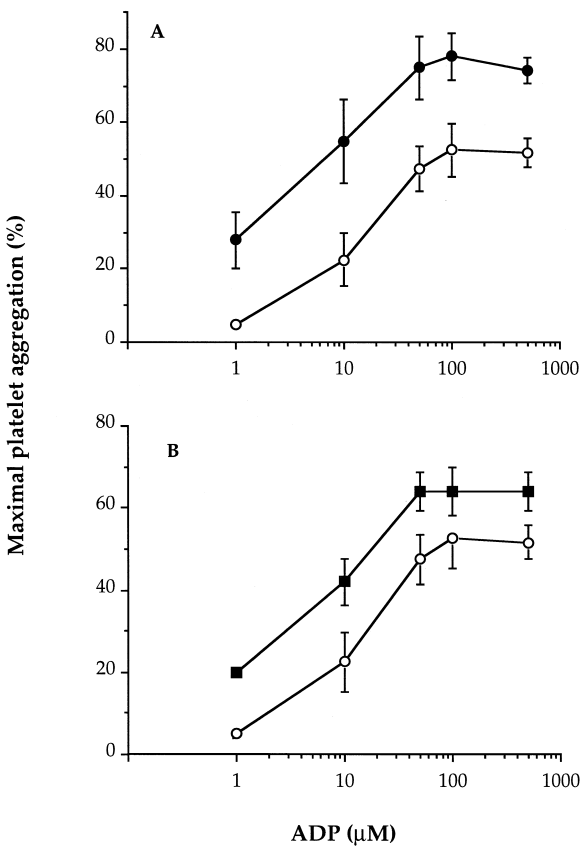

Fig. 3. The potentiation of ADP-induced platelet aggregation by ADP, adrenaline and 5-HT in washed platelets in the presence of aspirin. (A) Platelet aggregation induced by ADP alone (open circles, $EC_{50} = 12.5$ μM) and ADP in the presence of adrenaline (10 μM) (closed circles, $EC_{50} = 2.6$ μM). Adrenaline alone caused no platelet aggregation. (B) Platelet aggregation induced by ADP alone (open circles, $EC_{50} = 12.5$ μM) and ADP in the presence of 5-HT (10 μM) (closed squares, $EC_{50} = 3.0$ μM). 5-HT alone caused no platelet aggregation. The experiments were performed with washed platelets in the presence of aspirin (100 μM) and platelet-free plasma (5 μl/ml). Data are the mean ± sem ($n = 7$).

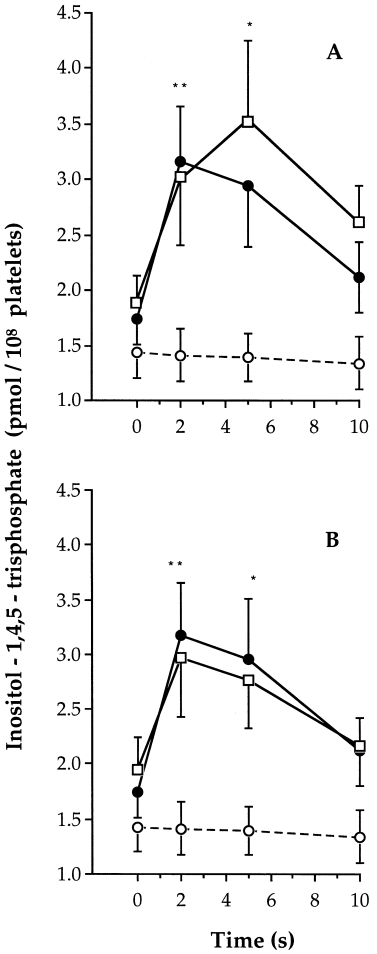

Fig. 4. The time-course of Ins(1,4,5)P_3 formation in washed human platelets stimulated by the combination of weak agonists in the presence of aspirin. (A) ADP 500 μM (filled circles), ADP in combination with adrenaline 10 μM (open squares) or buffer control (open circles). (B) ADP 500 μM (filled circles), ADP in combination with 5-HT 10 μM (open squares) or buffer control (open circles). The experiments were performed in the presence of aspirin (100 μM) and platelet-free plasma (5 μl/ml as a fibrinogen source). Data are the mean ± sem ($n = 11$, with duplicate determinations, in a randomised block design with respect to stimulus and time). $**$ ($P < 0.01$) and $*$ ($P < 0.05$) indicate a significant increase in Ins(1,4,5)P_3 production compared to buffer control at respective time points.

3.3. Ins(1,4,5)P_3 production by the weak agonists in combination

Adrenaline (10 μM) (Fig. 4A) or 5-HT (10 μM) (Fig. 4B) had no effect on the amount of Ins(1,4,5)P_3 generation by ADP (500 μM). The complete concentration response relationship for ADP-induced Ins(1,4,5)P_3 generation in the presence of adrenaline or 5-HT was then studied to investigate the mechanism involved in the potentiated aggregation response between the weak stimuli (Table 1). Ins(1,4,5)P_3 generation induced by ADP alone showed a consistent decrease at 100 μM, compared to 50 μM and 500 μM ADP. Adrenaline (1 μM) caused a small extra increase in the Ins(1,4,5)P_3 generated by ADP, which was only significant at 500 μM ADP, but not greater than the additive effect of the two agonists. Adrenaline alone also showed a significant production of Ins(1,4,5)P_3, as in Fig. 1B. 5-HT alone produced a significant effect on Ins(1,4,5)P_3 generation (Table 1 and Fig. 1C) but did not significantly increase Ins(1,4,5)P_3 generation by ADP. The ADP concentration–response relationship for Ins(1,4,5)P_3 generation (approximate EC_{50} 50 μM) was not shifted to the left in the presence of adrenaline or 5-HT (Table 1). Therefore there was no significant synergistic effect by adrenaline or 5-HT on stimulation of Ins(1,4,5)P_3 production by ADP.

3.4. Calcium mobilisation

Platelet aggregation and intracellular Ca^{2+} mobilisation responses stimulated by ADP alone and in combination with adrenaline in the presence and absence of aspirin are shown in Table 2. The aequorin loading procedure has caused some attenuation of the aggregation response compared to the experiments with washed platelets not loaded with aequorin. Nevertheless, adrenaline caused potentiation of platelet aggregation (Table 2A) which, in the absence of aspirin, was accompanied by a potentiation of ADP-induced Ca^{2+} mobilisation by adrenaline (Table 2A).

Table 2
Intracellular Ca^{2+} changes and maximal platelet aggregation induced by ADP and adrenaline in aequorin loaded platelets, in the presence (B) and absence of aspirin (A)

	ADP (μM)	Change in Ca^{2+} (μM)		Platelet aggregation (%)	
		ADP	ADP + adrenaline	ADP	ADP + adrenaline
A	10 μM	1.46 ± 0.12	2.25 ± 0.32[a]	3.3 ± 1.7	9.0 ± 2.7
	20 μM	2.2 ± 0.44	3.15 ± 0.27[a]	5.5 ± 2.8	18.0 ± 3.2[a]
	50 μM	1.98 ± 0.29	3.0 ± 0.46[a]	12.5 ± 3.5	30.0 ± 2.0[a]
	200 μM	2.65 ± 0.35	3.75 ± 0.38[a]	11.0 ± 3.0	48.3 ± 4.7[a]
	500 μM	2.65 ± 0.33	3.50 ± 0.33[a]	16.30 ± 3.8	55.8 ± 8.4[a]
B	20 μM	2.38 ± 0.13	2.51 ± 0.20	6.0 ± 2.0	23.5 ± 2.5[a]
	400 μM	2.62 ± 0.34	2.48 ± 0.41	20.8 ± 5.3	51.8 ± 5.1[a]
	500 μM	3.37 ± 0.71	3.77 ± 0.21	27.6 ± 6.5	66.0 ± 4.1[a]

Adrenaline concentration was 10 μM, which alone caused no platelet aggregation or mobilisation of Ca^{2+}. Two separate sets of experiments were conducted, one in the presence of aspirin (B) and one in the absence of aspirin (A). The data are mean ± sem from 5 experiments.
[a] Indicates a significant change ($P < 0.05$) in intracellular Ca^{2+} or platelet aggregation compared to the response for ADP alone.

Adrenaline alone caused no platelet aggregation or Ca^{2+} mobilisation (data not shown). In the presence of aspirin, the interaction between adrenaline and ADP was still able to achieve a potentiation of platelet aggregation (Table 2B) but this was not accompanied by a potentiated Ca^{2+} mobilization response (Table 2B). Thus, while platelet aggregation induced by ADP is potentiated by adrenaline (10 μM) both in the presence and absence of aspirin, Ca^{2+} mobilisation is potentiated only in the absence of aspirin (ie in the presence of thromboxane).

4. Discussion

The results from the present study show that generation of Ins(1,4,5)P_3 and therefore PtdIns(4,5)P_2 hydrolysis are stimulated by the weak agonists ADP, adrenaline and 5-HT. The failure of some previous studies to demonstrate the stimulation of the phosphoinositide pathway by these agonists (Fisher et al., 1985; MacIntyre et al., 1985) probably relates to a lack of assay sensitivity and a failure to document that the platelets have been isolated in such a way as to preserve their aggregation response, particularly to ADP. This is important because, in contrast to the effects of stronger stimuli, the aggregation response to ADP in particular is readily lost if the method for preparing suspensions of platelets washed free of plasma is not appropriate (Mustard et al., 1989).

We showed a significant increase in Ins(1,4,5)P_3 formation 2 s after ADP stimulation in a stirred aggregation system. This is in agreement with studies in a quenched-flow system (Raha et al., 1993) in which an increase in Ins(1,4,5)P_3 occurred at 1.5–5 s after the addition of ADP or thrombin and correlated with the mobilisation of intracellular Ca^{2+} within 2 s. In the quenched-flow system, an increase in Ins(1,4,5)P_3 was also found at 200 ms but the shear forces alone experienced by the platelets in this system were reported to cause the liberation of inositol phosphates (Raha et al., 1993). By contrast, in our experiments, there was no increase in Ins(1,4,5)P_3 caused by stirring alone in the absence of agonist.

The dose–response relationship for ADP-induced Ins(1,4,5)P_3 formation consistently showed a biphasic response (100 μM ADP compared to 50 μM and 500 μM, Table 1), occurring in all seven individual experiments. This is possibly explained by the high and low affinity states of the ADP receptor on the platelet membrane (Jefferson et al., 1988). Studies in other cells have reported a similar phenomenon (Kroegel et al., 1991).

The small but significant increase in mass of Ins(1,4,5)P_3 2 s after stimulation with adrenaline is the first demonstration of this in aspirin-treated human platelets and supports a very early study of adrenaline-stimulated phosphoinositide turnover in the presence of aspirin (Deykin and Snyder, 1973). The current view has been that adrenaline does not induce PtdIns(4,5)P_2 hydrolysis in platelets, because many others have been unable to demonstrate this using indirect methods with radioactive precursor incorporation or when thromboxane production is blocked by aspirin (Siess et al., 1984; MacIntyre et al., 1985; Drummond and MacIntyre, 1987). The α-adrenoreceptor on platelets is of the α_2-C10 subtype, which is coupled to PtdIns(4,5)P_2 hydrolysis in other cells (Kobilka et al., 1987; Cotecchia et al., 1990), lending further support to the findings in the present study.

In early studies 5-HT was shown to stimulate inositol 1-phosphate accumulation in rabbit platelets (Schächter et al., 1985). It has also been shown to induce a decrease in radiolabelled PtdIns(4,5)P_2 and an increase in diacylglycerol, although there was not a convincing concomitant increase of inositol 1,4-bisphosphate and Ins(1,4,5)P_3 (De Chaffoy de Courcelles et al., 1985). In both these previous reports, the experiments did not include a cyclooxygenase inhibitor. This present study is the first to report that 5-HT stimulates Ins(1,4,5)P_3 in platelets in the presence of aspirin at early time points.

The actions of weak agonists are mainly of importance when they act in synergism with other stimuli released in vivo from stimulated platelets into the milieu of developing haemostatic plugs or thrombi. Having determined the ability of the weak agonists to stimulate Ins(1,4,5)P_3 individually, we proceeded to determine whether the synergistic platelet aggregation between combinations of agonists occurs as the result of prior synergistic hydrolysis of PtdIns(4,5)P_2. The criterion to identify true synergism was the demonstration of a parallel shift to the left of the dose response curve to one agonist in the presence of another, for Ins(1,4,5)P_3 generation or platelet aggregation, as in our earlier study (Vanags et al., 1992), rather than using selected single doses of agonist. Adrenaline and 5-HT synergistically enhanced the ADP-induced platelet aggregation responses with a decrease in EC_{50}. However, synergistic enhancement of ADP-induced Ins(1,4,5)P_3 formation was not apparent, in the presence of adrenaline or 5-HT. Therefore no evidence of potentiated PtdIns(4,5)P_2 hydrolysis by these agents was found. Lalau-Keraly et al. (1987) also found that adrenergic potentiation of ADP aggregation did not involve changes in PtdIns(4,5)P_2, but Ins(1,4,5)P_3 was not measured.

The dose–response relationship for ADP-induced intracellular Ca^{2+} mobilisation in our study was potentiated in the presence of adrenaline for all concentrations of ADP in the absence of aspirin. This is in agreement with Potevin et al. (1990) who showed a similar potentiation, although only with a single concentration of ADP. In the presence of aspirin, however, we showed that adrenaline potentiated only ADP-induced platelet aggregation, but not intracellular Ca^{2+} mobilisation. It was not our intention to precisely correlate the dose of stimulus which caused Ins(1,4,5)P_3 generation and Ca^{2+} mobilisation, and thus the experiments were not designed to answer this question. We were more interested in whether either response, which

occurred earlier than platelet aggregation, showed similar synergism.

The evidence presented in our study strongly suggests that the intraplatelet mechanism of synergism between the weak agonists does not involve PtdIns(4,5)P_2 hydrolysis or Ca^{2+} mobilisation when thromboxane is inhibited, at least not at early (< 10 s) time points. The lack of synergism at the signal transduction level suggests that the pathways for the stimulation of the platelet aggregation response remain separate at least as far as the formation of Ins(1,4,5)P_3 and Ca^{2+} mobilisation. It seems likely that it is only after these steps that the pathways interact to produce synergism. This result is of considerable interest. Conceptually, at the stage of first interaction of a stimulus with the platelet surface, each of the stimuli acts via a different pathway which subsequently converges with the pathways for other stimuli at a critical point in the signal transduction cascade. It would be of interest to know if this was also true for some of the other stimuli such as thromboxane, collagen and thrombin. It may then be possible to design specific platelet inhibitors, which act at this critical point in the activation cascade.

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