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Synergism between thrombin and adrenaline (epinephrine) in human platelets

Marked potentiation of inositol phospholipid metabolism

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We have studied synergism between adrenaline (epinephrine) and low concentrations of thrombin in gel-filtered human platelets prelabelled with [^{32}P]P_i. Suspensions of platelets, which did not contain added fibrinogen, were incubated at 37 °C to measure changes in the levels of ^{32}P-labelled phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-phosphate (PIP) and phosphatidate (PA), aggregation and dense-granule secretion after stimulation. Adrenaline alone (3.5–4.0 μM) did not cause a change in any parameter (phosphoinositide metabolism, aggregation and dense-granule secretion), but markedly enhanced the thrombin-induced responses over a narrow range of thrombin concentrations (0.03–0.08 units/ml). The thrombin-induced hydrolysis of inositol phospholipids by phospholipase C, which was measured as the formation of [^{32}P]PA, was potentiated by adrenaline, as was the increase in the levels of [^{32}P]PIP_2 and [^{32}P]PIP. The presence of adrenaline caused a shift to the left for the thrombin-induced changes in the phosphoinositide metabolism, without affecting the maximal levels of ^{32}P-labelled compounds obtained. A similar shift by adrenaline in the dose–response relationship was previously demonstrated for thrombin-induced aggregation and dense-granule secretion. Also, the narrow range of concentrations of thrombin over which adrenaline potentiates thrombin-induced platelet responses is the same for changes in phosphoinositide metabolism and physiological responses (aggregation and dense-granule secretion). Our observations clearly indicate that adrenaline directly or indirectly influences thrombin-induced changes in phosphoinositide metabolism.

INTRODUCTION

Platelets respond to thrombin stimulation by a rapid turnover of the inositol phospholipids (PIP_2, PIP and PI) through phospholipase C (PLC) activation, with the corresponding formation of inositol phosphates (DG and PA) (for reviews, see Berridge, 1985, 1986; Abdel-Latif, 1986; Steen & Holmsen, 1987). Also, there is an influx and mobilization of cytoplasmic Ca^{2+}, phosphorylation of various proteins and alkalinization of the intracellular pH (via activation of Na^+/H^+ exchange). DG and IP_3 are believed to be stimulatory intracellular messengers working via activation of protein kinase C and mobilization of intracellular Ca^{2+} respectively. Concurrent with these putative signal-processing mechanisms, platelets display a sequence of distinct physiological responses, including shape change, primary and secondary aggregation, three different types of secretion (from dense granules, alpha granules and lysosomes) and arachidonate liberation.

In contrast with most other platelet agonists, adrenaline (epinephrine) induces aggregation and dense-granule secretion without a preceeding shape change; the presence of fibrinogen and low concentrations of Ca^{2+} is required in the incubating suspension (Patscheke, 1980; Plow & Marguerie, 1980; Lages & Weiss, 1981). The adrenergic receptors in human platelets are mainly of the $alpha_2$ subclass (Grant & Scrutton, 1979). At present, adrenaline is in itself proposed to induce only a few directly receptor-mediated effects in platelets, namely inhibition of adenylate cyclase, intracellular alkalinization and mobilization of arachidonate via an Na^+/H^+-exchange-dependent phospholipase A_2 activation and probably, primary aggregation (including exposure of fibrinogen receptors) (Jakobs *et al.*, 1976; Connolly & Limbird, 1983; Sweatt *et al.*, 1985, 1986*a*,*b*; Banga *et al.*, 1986). The other effects and responses observed, such as PLC activation, secondary aggregation and secretion, are believed to occur indirectly, e.g., through arachidonate liberation and formation of cyclo-oxygenase products (stimulatory prostaglandins and thromboxanes) (Siess *et al.*, 1984; Sweatt *et al.*, 1986*a*,*b*; Banga *et al.*, 1986). It is important to note that platelet responsiveness to adrenaline alone may vary greatly between different donors (O'Brien, 1964).

Two platelet agonists may, when added together in low concentrations, potentiate the effect of each other. Such a potentiation of the response is termed 'synergistic' if it is more than additive. For adrenaline and thrombin, such a synergistic action has been shown for optical aggregation (O'Brien, 1964; Thomas, 1967), dense-granule secretion (Steen & Holmsen, 1985) and mobilization of cytoplasmic Ca^{2+} (Ware *et al.*, 1987; Ardlie *et al.*, 1987). Here we report that adrenaline potentiates thrombin-induced [^{32}P]PA formation and changes in the levels of [^{32}P]PIP_2 and [^{32}P]PIP in human gel-filtered

Abbreviations used: DG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidate; P_i, inorganic orthophosphate; PLC, phospholipase C; a unit of thrombin activity is expressed in NIH (National Institutes of Health) units (nominal) according to the NIH assay procedure.

platelets, and that this potentiation is not blocked by the cyclo-oxygenase inhibitor indomethacin.

MATERIALS AND METHODS

Stock solutions of adrenaline (epinephrine; Sigma, St. Louis, MO, U.S.A.) and bovine thrombin (Hoffmann–La Roche, Basel, Switzerland) were prepared in 0.15 M-NaCl and stored in the dark at −20 °C in small portions. Appropriate dilutions were made in 0.15 M-NaCl immediately before use, and kept on ice during the experiment. Indomethacin (Sigma) was dissolved in ethanol; the final concentration of ethanol in the incubation mixtures was 0.05%. All concentrations mentioned in the Results section are final concentrations.

Human blood treated with 0.15 vol. of the anti-coagulant ACD-buffer (85 mM-trisodium citrate/111 mM-dextrose/71 mM-citric acid) was centrifuged at 535 *g* for 6 min. The supernatant platelet-rich plasma (PRP) was then centrifuged at 1200 *g* for 10 min. The upper two-thirds of the supernatant were discarded, and the pellet of platelets resuspended in the remainder of the plasma. The concentrated PRP was incubated at 37 °C for 60 min with 0.1 mCi of [^{32}P]P_i (carrier-free; code no. PBS 11; Amersham International)/ml. The platelets were then gel-filtered as described previously (Steen & Holmsen, 1985). Platelet numbers were determined with a Coulter counter (model ZM, Luton, Beds., UK.). The suspension of gel-filtered platelets was adjusted to 3.5×10^8 platelets/ml. Suspensions of these pulse-labelled platelets were incubated at 37 °C with adrenaline, bovine thrombin (or both) or 0.15 mM-NaCl. When using both thrombin and adrenaline, adrenaline was added 10 s before thrombin.

Platelet aggregation was measured in a Payton Associates dual-channel aggregation module connected to a linear two-channel recorder. The samples (7.0 ml) were stirred at 800 rev./min in cuvettes and absorbance recorded at a chart speed of 2 cm/min.

Dense-granule secretion was measured as the extracellular appearance of ADP plus ATP. Samples (0.2 ml) were transferred from the incubation mixture into 0.14 vol. of 1 M-formaldehyde/50 mM-EDTA on ice. Further treatment and the method for determination of the content of ADP plus ATP was described previously (Steen & Holmsen, 1985). The extent of secretion was expressed as a percentage of the total content of these nucleotides in the platelets.

Samples (0.5 ml; 1 vol.) for determination of phospholipid radioactivity were withdrawn from the incubating suspension and mixed with 4.0 vol. of chloroform/methanol/conc. HCl (20:40:1, by vol.; 0 °C). Water (1.0 vol.) and chloroform (1.0 vol.) were added with vigorous shaking; the samples were then processed further as described previously (Holmsen *et al.*, 1984). Separation of phospholipds was by t.l.c. at room temperature on silica-gel plates (Merck; art 5553) pretreated with 1% potassium oxalate. As solvent systems, the methylamine system with chloroform/methanol/20% (v/v) methylamine (30:18:5, by vol.) or acetone system with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) were used. The spots were localized by overnight radioautography with Fuji X-ray film, then scraped off and the ^{32}P-content of the phospholipids was determined by liquid-scintillation counting. The results are expressed as a percentage of the values for saline-only-treated controls.

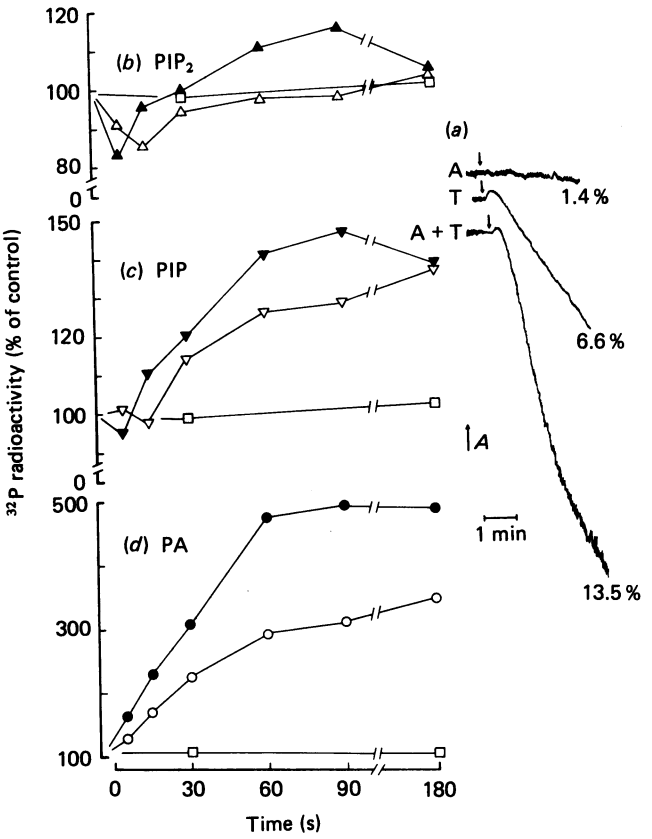

Fig. 1. Synergistic effect of adrenaline on (*a*) thrombin-induced optical aggregation and dense-granule secretion and (*b–d*) changes in time of PIP_2, PIP and PA metabolism

(*a*) Gel-filtered, [^{32}P]P_i-labelled platelets were stirred at 800 rev./min at 37 °C in a two-channel aggregometer. Adrenaline (A; 3.5 μM) or 0.15 M-NaCl was added 10 s before the stimulation with thrombin (T; 0.05 unit/ml) or 0.15 M-NaCl (the latter addition is indicated by an arrow on the aggregation curves). A constant volume (0.2 ml) was withdrawn from the reaction samples after 180 s of incubation to measure secretion, which is expressed as percentage of total platelet ADP+ATP content (result indicated beside the corresponding aggregation tracing). (*b–d*) In the same platelet suspension, samples (0.5 ml) were withdrawn at the times shown to measure changes in the ^{32}P radioactivity in (*b*) PIP_2 (△, ▲), (*c*) PIP (▽, ▼) and (*d*) PA (○, ●); open symbols indicate stimulation with thrombin alone, closed symbols adrenaline plus thrombin. No changes took place when the platelets were incubated with adrenaline alone (□). The data are means for two identical independent experiments.

RESULTS

Fig. 1 shows the time course of thrombin-induced changes in the ^{32}P radioactivity of the inositol phospholipds and the corresponding optical aggregation and dense-granule secretion (end point at 180 s) in the absence or presence of adrenaline in [^{32}P]P_i-prelabelled platelets. The results for phosphoinositide metabolism are means for two independent experiments, which were

chosen because of their approximately similar synergistical aggregation tracings and degrees of secretion. Under the experimental conditions used (Ca^{2+}-free medium, no added fibrinogen), adrenaline alone (3.5 μM) did not cause any change in the parameters measured (Fig. 1).

Thrombin alone (0.05 unit/ml) induced a progressive formation of [^{32}P]PA (to approx. 310% of control at 90 s). In the presence of adrenaline, the thrombin-induced production of [^{32}P]PA was markedly enhanced both with respect to the initial velocity and the final extent (approx. 500% of control after 90 s) as compared with the response obtained with thrombin alone. With thrombin alone, the level of [^{32}P]PIP_2 decreased initially, followed by a very slow recovery to the basal level. The [^{32}P]PIP radioactivity increased gradually above the initial level and reached approx. 125% of control at 90 s. In the presence of adrenaline the [^{32}P]PIP_2 level also decreased initially, but was followed by an increase which proceeded to above control levels to reach a maximal value after 90 s of incubation. Thus the addition of adrenaline lead to more extensive thrombin-induced changes in the [^{32}P]PIP_2 level. Similarly, the thrombin-induced increase in the level of [^{32}P]PIP up to 90 s was more marked in the presence of adrenaline (approx. 145% of control).

Concomitantly, thrombin alone (0.05 unit/ml) only induced a slow aggregation response and a sluggish secretion of ADP plus ATP (Fig. 1). In the presence of adrenaline (3.5 μM), both thrombin-induced aggregation and dense-granule secretion were potentiated more than 2-fold.

The results given in Fig. 1 were obtained with stirring of the platelet suspensions, which allows optical aggregation to occur. Also, the time courses only show data obtained with one concentration of thrombin. Therefore we decided to examine the thrombin-induced changes in the metabolism of the phosphoinositides under non-aggregating conditions (no stirring) over a range of concentrations of thrombin in the absence and presence of adrenaline; this was done to determine the range of concentrations of thrombin over which synergistic potentiation of the phosphoinositide turnover occurs. An incubation time of 90 s was chosen; at this time of incubation, the changes in the levels of [^{32}P]PA, [^{32}P]PIP_2 and [^{32}P]PIP were most extensive (see Fig. 1).

Fig. 2 shows the dose–response relationships at 90 s of incubation for the thrombin-induced changes in the ^{32}P radioactivity of the phosphoinositides with or without adrenaline in an unstirred system. The data are means ± S.D. for six determinations obtained in two independent experiments performed in triplicate. As in the stirred system, adrenaline alone (4 μM) did not cause a change in the radioactivity of any of the phospholipids measured. When the concentration of thrombin alone was increased from 0.01 to 0.15 unit/ml, the amount of [^{32}P]PA formed at 90 s gradually increased, from approx. 115% to approx. 1470% of the basal level in unstimulated platelets. This increased ^{32}P labelling of PA indicates PLC activation. With thrombin alone, the [^{32}P]PIP_2 level at 90 s was significantly lower than the control level at 0.01 and 0.03 unit of thrombin/ml, and close to the control level at 0.05 unit/ml. Compared with the time courses shown in Fig. 1, these results indicate that low concentrations of thrombin induce an initial decrease in the [^{32}P]PIP_2 level, but after this phase there

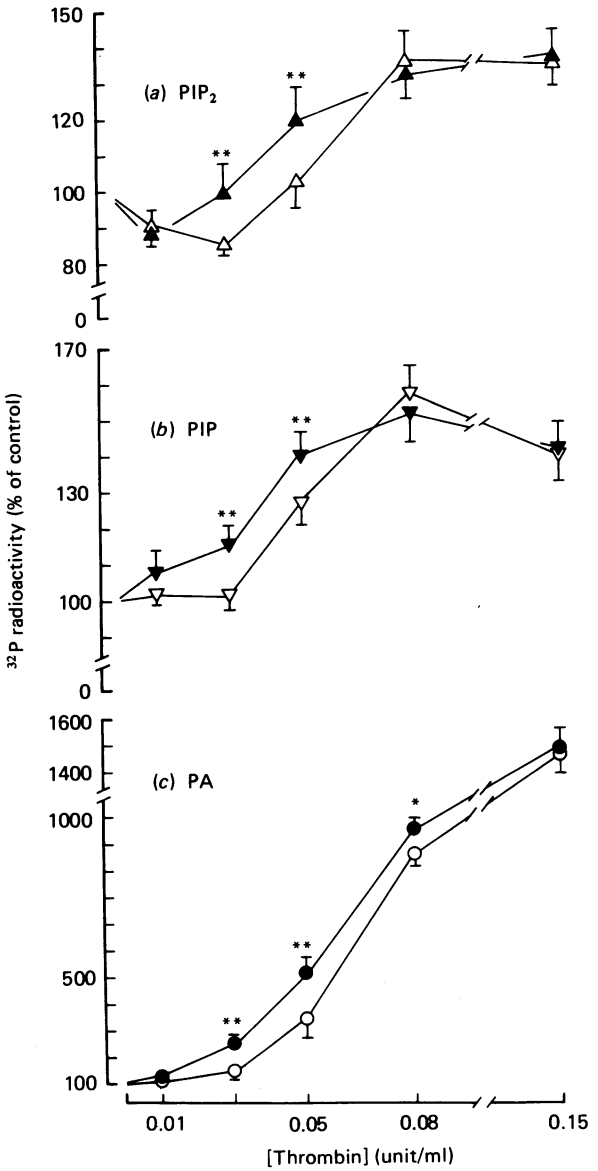

Fig. 2. Synergistic effect of adrenaline on changes in ^{32}P radioactivity of (a) PIP_2, (b) PIP and (c) PA after 90 s induced by various concentrations of thrombin

Gel-filtered [^{32}P]P_i-labelled platelets were incubated in a water bath at 37 °C without stirring. Adrenaline (4.0 μM) or 0.15 M-NaCl was added 10 s before thrombin or 0.15 M-NaCl. A constant volume (0.5 ml) was withdrawn after 90 s of incubation to measure the changes in the ^{32}P radioactivity of (*a*) PIP_2 (△, ▲), (*b*) PIP (▽, ▼) and (*c*) (○, ●); open symbols indicate stimulation with thrombin alone, closed symbols indicate adrenaline (4.0 μM) plus thrombin. The results are means ± S.D. for six determinations obtained in two independent identical experiments performed in triplicate. The asterisks indicate the paired samples for which adrenaline significantly ($*P < 0.05$; $**P < 0.025$) increased the thrombin-induced changes in the ^{32}P radioactivity of the phospholipids. The statistical significance was determined by using the paired Student's *t* test.

is only a very slow further increase. At higher concentrations, however, an increase to approx. 135% was measured. The [^{32}P]PIP level was close to controls at 0.01 and 0.03 unit of thrombin/ml, but increased

Table 1. Effect of the cyclo-oxygenase inhibitor indomethacin on thrombin-induced [^{32}P]PA formation potentiated by adrenaline

The experimental procedure given in Fig. 2 was used. The final concentration of indomethacin was 20 μM. The data are means of 5 determinations obtained in 3 independent experiments. The statistical significance, P (thrombin versus adrenaline + thrombin), was determined by using the paired Student's t test.

Treatment	^{32}P radioactivity (% of control)			
	Saline		Indomethacin	
Thrombin (0.03 unit/ml)	168	$P < 0.05$	191	$P < 0.05$
Thrombin (0.03 unit/ml) + adrenaline (4.0 μM)	225		251	
Thrombin (0.05 unit/ml)	313	$P < 0.05$	322	$P < 0.05$
Thrombin (0.05 unit/ml) + adrenaline (4.0 μM)	447		461	

markedly with the increase in thrombin concentration, reaching approx. 175% at 0.08 unit of thrombin/ml.

The level of [^{32}P]PA at 90 s was significantly increased by adrenaline (4.0 μM) at 0.03, 0.05 and 0.08 unit of thrombin/ml ($P < 0.025$, 0.010 and 0.050 respectively), as compared with the levels obtained with thrombin alone. Also, the [^{32}P]PIP_2 level obtained with thrombin at 90 s was significantly increased with adrenaline at 0.03 and 0.05 unit of thrombin/ml ($P < 0.025$ and 0.010 respectively). At higher concentrations no potentiation by adrenaline was observed. Therefore, over the thrombin concentration range tested, adrenaline only increased the [^{32}P]PIP_2 level at low thrombin concentrations and did not increase the maximal level of PIP_2 obtained at the high concentrations. Similarly, the [^{32}P]PIP level was significantly elevated by the presence of adrenaline at 0.03 and 0.05 unit of thrombin/ml ($P < 0.005$ and 0.010 respectively), without an increase in the maximal level obtained with higher concentrations of thrombin. The level of [^{32}P]PI increased gradually with increasing concentrations of thrombin alone, and was markedly potentiated by adrenaline at 0.03 and 0.05 unit of thrombin/ml (results not shown).

Concomitantly, platelet secretion was measured in the same incubation samples as an indicator of a physiological platelet response. Synergistic potentiation by adrenaline on dense-granule secretion was only seen when induced by 0.03 and 0.05 unit of thrombin/ml (11.4% versus 8.3%, 28.2% versus 14.2% with or without adrenaline respectively); i.e., over the same range of thrombin concentrations as that over which synergistical changes in the phosphoinositide metabolism were observed.

To determine the importance of the cyclo-oxygenase pathway in thrombin-induced [^{32}P]PA formation potentiated by adrenaline, indomethacin was used in some experiments to block the cyclo-oxygenase activity. As shown in Table 1, the addition of 20 μM-indomethacin did not inhibit adrenaline's enhancement of thrombin-induced [^{32}P]PA production; neither was the synergistically induced dense-granule secretion blocked (results not shown).

DISCUSSION

Both previous and recent studies (O'Brien, 1964; Thomas, 1967; Steen & Holmsen, 1985) have clearly demonstrated that adrenaline synergistically potentiates thrombin-induced optical aggregation and dense-granule secretion in human platelets. This potentiation, however, only occurs within a narrow range of low concentrations of thrombin (Steen & Holmsen, 1985), i.e., concentrations that have rarely been used in previous studies of the phosphoinositide metabolism.

Therefore the aim of the present investigation was to determine whether or not the synergistical potentiation by adrenaline observed for thrombin-induced physiological platelet responses (optical aggregation and dense-granule secretion) was also parallelled by the agonist-induced changes in phosphoinositide metabolism. In the present work, we show for the first time that (1) adrenaline potentiates changes in [^{32}P]phosphoinositide metabolism induced by low concentrations of thrombin in gel-filtered human platelets, and (2) the presence of adrenaline causes a shift to the left of the dose–response curve for the thrombin-induced changes.

Adrenaline alone induced no changes in any parameter measured under the experimental conditions used (Ca^{2+}-free medium with no added fibrinogen). These results are in accordance with previously obtained data establishing that adrenaline requires extracellular bivalent cations (Ca^{2+}) and the presence of fibrinogen to induce fibrinogen-receptor exposure and aggregation (Plow & Marguerie, 1980; Patscheke, 1980; Lages & Weiss, 1981). Also, adrenaline does not by itself directly activate phospholipase C (Siess *et al.*, 1984; Banga *et al.*, 1986), but may indirectly cause phosphoinositide breakdown by the PLC pathway via arachidonic acid metabolites (see the Introduction). Therefore both PLC activation and aggregation induced by adrenaline alone in human platelets are blocked by the cyclo-oxygenase inhibitors indomethacin and acetylsalicylic acid (Patscheke, 1980; Siess *et al.*, 1984; Banga *et al.*, 1986).

The presence of adrenaline clearly potentiates thrombin-induced changes in [^{32}P]phosphoinositide metabolism, aggregation and dense-granule secretion, and causes a shift to the left of the dose–response curve for the thrombin-induced changes in the ^{32}P radioactivity in PIP_2, PIP and PA. A similar shift in the dose–response curve by adrenaline has previously been demonstrated for thrombin-induced aggregation and dense-granule secretion (Steen & Holmsen, 1985). Also, the narrow range of concentrations of thrombin within which adrenaline causes synergistic potentiation of the responses are the same for phosphoinositide hydrolysis and physiological platelet responses (aggregation and dense-granule secretion; Steen & Holmsen, 1985).

The formation of [^{32}P]PA is established to be an indirect, but sensitive, indicator of PLC activation (Banga *et al.*, 1986; Bushfield *et al.*, 1987; Lapetina & Siess, 1987). Our results may therefore indicate that adrenaline enhances the thrombin-induced degradation of inositol phospholipids by PLC. These observations are in accordance with some recent reports that adrenaline potentiates hydrolysis of inositol phospholipids induced by ADP, vasopressin or 5-hydroxytryptamine (serotonin) in platelets (Bushfield *et al.*, 1987; de Chaffoy de Courcelles *et al.*, 1987; Lalau Keraly *et al.*, 1987). At present, however, it is not clear whether the potentiating effect of adrenaline on agonist-induced changes in PI metabolism is a direct action at the PLC or G-protein level, or an indirect effect through mechanisms such as arachidonate liberation, inhibition of the adenylate cyclase system etc. Also, the interpretation of the data demonstrating changes in the levels of [^{32}P]PIP_2 and [^{32}P]PIP is very complex. Our results may, however, indicate that adrenaline influences the activity of the polyphosphoinositide kinase and phosphatase pathways in thrombin-stimulated platelets.

PLC activation induced by adrenaline alone is, as already mentioned, blocked by cyclo-oxygenase inhibitors. The potentiating effect of adrenaline on thrombin-induced phosphoinositide hydrolysis and dense-granule secretion is, however, not inhibited by indomethacin (Table 1). It has been demonstrated that adrenaline synergistically enhances thrombin-induced mobilization of cytoplasmic Ca^{2+} in platelets (Thompson *et al.*, 1986; Ardlie *et al.*, 1987; Ware *et al.*, 1987), and this potentiating effect of adrenaline is not inhibited by acetylsalicylic acid (Ware *et al.*, 1987). Similarly, adrenaline enhances fibrinogen-receptor exposure and aggregation of aspirin-treated platelets stimulated with thrombin (Peerschke, 1984). Therefore, it seems likely that the potentiating (but not the direct) effect of adrenaline on thrombin-induced platelet activation is independent of the cyclo-oxygenase pathway. However, it has recently been demonstrated that arachidonic acid by itself may induce Ca^{2+} mobilization (Wolf *et al.*, 1986). Therefore, it is possible that an adrenaline-induced arachidonate liberation may explain why the potentiating effects of adrenaline are independent of the cyclo-oxygenase pathway.

It is clearly established that adrenaline may decrease an already elevated level of intracellular cyclic AMP in human platelets (Robison *et al.*, 1969; Jakobs *et al.*, 1976; Aktories & Jakobs, 1981). Some previous studies have shown that the level of intracellular free Ca^{2+} in platelets may be regulated through the level of cyclic AMP (Feinstein *et al.*, 1983; Yamanishi *et al.*, 1983; Zavoico & Feinstein, 1984). Since Ca^{2+} is an intracellular messenger, and its cytoplasmic mobilization is synergistically potentiated by adrenaline, an effect on the intracellular cyclic AMP level could in part explain the action of adrenaline. It has, however, been reported that alpha$_2$-adrenergic mediated inhibition of prostaglandin E_1-stimulated cyclic AMP accumulation occurred normally in platelets desensitized to adrenaline-induced aggregation (Motulsky *et al.*, 1986). Also, there has been shown to be an apparent dissociation between the effects of extracellular Na^+ removal on α-adrenergic-induced human platelet functional responses and α-adrenergic inhibition of cyclic AMP accumulation (Connolly & Limbird, 1983). In addition, the potentiating effect of adrenaline is not mimicked by 2′,5′-dideoxyadenosine, which, like adrenaline, inhibits adenylate cyclase (Thompson *et al.*, 1986). These data may indicate that the synergistic enhancement by adrenaline of platelet responses induced by other agents does not involve adenylate cyclase inhibition.

It has been suggested that the exposure of fibrinogen receptors and platelet aggregation induced by adrenaline are responses partly or totally dependent on the presence of ADP, a well-known platelet agonist (Plow & Marguerie, 1980; Connolly & Limbird, 1983; Lanza & Cazenave, 1985; Figures *et al.*, 1986). Recently it has been shown that the ADP-scavenger system creatine phosphate/creatine phosphokinase did not prevent the enhancement by adrenaline of thrombin-induced aggregation and cytoplasmic Ca^{2+} mobilization; the potentiating effect was, conversely, diminished (Ware *et al.*, 1987). Preliminary data obtained in our laboratory have indicated that the potentiating effect of adrenaline on thrombin-induced changes in PI metabolism still obtains in a cyclo-oxygenase-inhibited system where all extracellular ADP is removed by creatine phosphate/creatine phosphokinase. Therefore the potentiating action of adrenaline on thrombin-induced platelet responses seems to be independent of cyclo-oxygenase metabolites and extracellular ADP.

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