Possible mechanisms of the potentiation of blood-platelet activation by adrenaline

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Vasopressin and adrenaline in combination exert synergistic effects on platelet activity. This study investigated the effects of sub-threshold concentrations of adrenaline (0.1–1 μM) on vasopressin (10 nM–1 μM)-induced platelet aggregation, ATP secretion, elevation of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and hydrolysis of inositol phospholipids, monitored as [^{32}P]phosphatidic acid formation. Potentiation of vasopressin-induced aggregation and ATP secretion by adrenaline was accompanied by enhanced elevation of $[\text{Ca}^{2+}]_i$ and [^{32}P]phosphatidic acid formation. The stimulatory effects of adrenaline on vasopressin-induced platelet activation were mimicked by the combination of the Ca^{2+} ionophore, ionomycin, and the protein kinase C activator, phorbol 12-myristate 13-acetate, but not by either of these agents alone. These results suggest that the potentiation of vasopressin-induced platelet activation by adrenaline is mediated via enhancement of inositol phospholipid hydrolysis and elevation of $[\text{Ca}^{2+}]_i$.

INTRODUCTION

In platelets, as in many other cell types, it has been established that inositol phospholipid hydrolysis serves as one of the transduction processes that link receptor occupancy to cellular activation. Phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate yields 1,2-diacylglycerol (DG), an activator of protein kinase C (Kaibuchi *et al.*, 1983), and inositol 1,4,5-trisphosphate which can mobilize Ca^{2+} ions from intracellular stores (Streb *et al.*, 1983; O'Rourke *et al.*, 1985). The involvement, if any, of inositol phospholipid hydrolysis in agonist-induced Ca^{2+} influx, also known to occur in platelets, remains to be determined. Elevation of the cellular content of DG and of the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) results in the synergistic stimulation of platelet (Rink *et al.*, 1983) and cellular reactivity (Michell, 1983; Berridge, 1984). Stimulation of platelet responsiveness by many agonists, for example thrombin, vasopressin (VP), platelet-activating factor and thromboxane A_2 mimetics, is associated with increased inositol phospholipid hydrolysis (Kaibuchi *et al.*, 1983; Brydon *et al.*, 1983) and elevation of $[\text{Ca}^{2+}]_i$ (Rink *et al.*, 1982; MacIntyre *et al.*, 1985). Adrenaline is anomalous among platelet agonists in that it fails to stimulate either inositol phospholipid hydrolysis or elevation of $[\text{Ca}^{2+}]_i$, at least as monitored by quin2 (Brydon *et al.*, 1983; Siess *et al.*, 1984; MacIntyre *et al.*, 1985; Ware *et al.*, 1986). Consequently it has been proposed that adrenaline activated platelets via an undefined mechanism(s), independent of inositol phospholipid hydrolysis and elevation of $[\text{Ca}^{2+}]_i$. More recently, this view has been challenged by the observations of Ware *et al.* (1986), who showed that adrenaline increased the luminescence of platelets loaded with the Ca^{2+} -sensitive photoprotein aequorin. This may indicate an increase in $[\text{Ca}^{2+}]_i$ that is

sufficiently small and/or sufficiently localized that it does not disturb the spatial average $[\text{Ca}^{2+}]_i$ which is monitored by quin2 (Blinks *et al.*, 1982; Johnson *et al.*, 1985). Platelet α_2 -adrenoceptors can be coupled to inhibition of adenylate cyclase activity. Whether adrenaline-induced platelet activation could result from a decrease in the basal concentration of cyclic AMP, perhaps in some specific pool, is controversial (Salzman, 1972; Haslam *et al.*, 1978).

Besides directly activating platelets when added at high concentrations (around 1–10 μM), adrenaline at sub-threshold concentrations can potentiate platelet responsiveness to a variety of other agonists. The aggregatory and secretory responses of platelets to agonists such as ADP, VP and platelet-activating factor can be markedly potentiated by prior or simultaneous addition of adrenaline (O'Brien, 1964; Ardlie *et al.*, 1966; Mills & Roberts, 1967; Nakanishi *et al.*, 1971; Michal & Motamed, 1976; Grant & Scrutton, 1980; Vargaftig *et al.*, 1982; Fouque & Vargaftig, 1984). Although thromboxane A_2 production and release of platelet granule constituents are not pre-requisite for this effect to occur (Rao *et al.*, 1980; Rao & White, 1981; Cameron & Ardlie, 1982; Fouque & Vargaftig, 1984), the mechanisms of this potentiation of agonist-induced platelet activation by adrenaline are largely unknown.

The aims of the present study were to investigate the effects of adrenaline on the transduction processes activated by VP and to examine the role of changes in $[\text{Ca}^{2+}]_i$ or DG in potentiation of VP-induced platelet activation by adrenaline. The former we addressed by monitoring the effects of prior or simultaneous addition of adrenaline on elevation of $[\text{Ca}^{2+}]_i$ and phosphoinositide hydrolysis induced by VP. The latter we assessed by comparing the effectiveness of the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), and

Abbreviations used: DG, 1,2-diacylglycerol; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; VP, vasopressin; PMA, phorbol 12-myristate 13-acetate; PtdA, phosphatidic acid; DMSO, dimethyl sulphoxide.

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Fig. 1. Effects of adrenaline on vasopressin-induced aggregation and ATP secretion

(a) Traces show typical aggregation responses from a single representative experiment. VP (3 nM) was added to stirred (900 rev./min) platelet suspensions (2×10^8 cells/ml, 37 °C) which had been incubated with saline or adrenaline (ADR; 0.1 μ M) for the times indicated. Prolonged incubation with adrenaline (0.1 μ M) did not result in any increase in light transmission (not shown). Inclusion of the cyclo-oxygenase inhibitor flurbiprofen (10 μ M) in the incubation medium did not affect the pattern of responsiveness. (b) The bars show effects of co-addition of, or preincubation for various time intervals with, adrenaline (0.1–1 μ M) on the initial rate of vasopressin-induced aggregation (□) and the degree of ATP secretion (■). To facilitate comparison between different experiments, results are expressed as a percentage of the response to vasopressin plus saline in each experiment. The results shown are means $\pm$ S.E.M. of three experiments using blood from different donors. Data were compared with the saline-treated control by Student's *t* test: **P* < 0.05, ***P* < 0.005. Similar results were obtained when adrenaline (0.1–1 μ M) was added in combination with submaximal concentrations of platelet-activating factor (1–18 nM).

the Ca^{2+} ionophore, ionomycin, alone and in combination, in mimicking the potentiatory effect of adrenaline on platelet responsiveness to VP.

METHODS

Blood was obtained from healthy volunteers who denied taking drugs known to affect platelet function. Blood was collected into 10% (v/v) trisodium citrate (0.13 M) and centrifuged (1000 *g* for 5 min; room temperature). The supernatant platelet-rich plasma was aspirated and, in most cases (except phospholipid studies) was incubated with 10–15 μ M-quin2 acetoxy-methyl ester (30–40 min; 37 °C). Quin2-labelled platelets were separated from plasma containing extraneous dye by gel filtration on columns of Sepharose 2B equilibrated with a modified HEPES-buffered Tyrode solution containing 0.35% bovine serum albumin (Pollock *et al.*, 1984). The albumin was omitted in studies with ionomycin. The

quin2 content of the platelets was around 1 mmol/litre of cell water. Gel-filtered platelets were stored in stoppered plastic tubes at 37 °C. Immediately before use the external Ca^{2+} concentration was adjusted either to 1 mM by addition of CaCl_2 or to around 10 nM by addition of EGTA. For the measurement of $[\text{Ca}^{2+}]_i$, platelets (2 ml; approx. 5×10^7 cells/ml) were placed in 1 cm-square quartz cuvettes at 37 °C and fluorescence was monitored in a Perkin-Elmer LS3 fluorescence spectrometer with standard monochromator settings of 339 nm excitation and 492 nm emission. Aggregation and ATP secretion were monitored simultaneously in stirred (700 rev./min) platelet samples (0.45 ml; 37 °C) by using a luminescence-aggregometer (Chronolog Corp.). Incubations were for the indicated times with adrenaline, PMA, ionomycin or vehicle (saline or DMSO) before the addition of a sub-maximal concentration of VP. The optimal agonist concentration (i.e. that which elicited around 10–30% of maximum response) was determined

Fig. 2. Effects of adrenaline on vasopressin-induced elevation of $[Ca^{2+}]_i$

(a) Traces show typical fluorescence recordings from a single representative experiment. Samples were incubated with saline or adrenaline (ADR; $0.1 \mu M$) for the times indicated before addition of VP (10 nM). Changes in $[Ca^{2+}]_i$ (indicated on the right) were calculated from the observed changes in dye fluorescence. Flurbiprofen ($10 \mu M$) did not affect the pattern of responsiveness. (b) Bars show mean data from seven different experiments. To facilitate comparison, data are expressed as percentage of the control (saline-treated) elevation of $[Ca^{2+}]_i$ above the resting value. Basal $[Ca^{2+}]_i$ was $90 \pm 3 \text{ nM}$ (mean $\pm$ S.E.M., $n = 7$). The control elevation in $[Ca^{2+}]_i$ (100% response) was $80 \pm 8 \text{ nM}$. Data were compared with saline-treated controls by Student's *t* test: * $P < 0.05$. Similar data were obtained with submaximal concentrations of platelet-activating factor ($1\text{--}18 \text{ nM}$) in combination with adrenaline ($0.1\text{--}5 \mu M$).

at the start of each experiment and varied from 3 to 30 nM-VP when albumin was present in the buffer, or around 10-fold lower concentrations when albumin was omitted.

Phospholipid studies were carried out essentially as described previously (Pollock *et al.*, 1984). Adrenaline or saline was added for the times indicated and reactions were initiated by addition of VP ($3\text{--}100 \text{ nM}$). After 30 s reactions were terminated (Pollock *et al.*, 1984) and lipids were extracted, dried at 40°C under N_2 , separated by two-dimensional t.l.c. (Yavin & Zutra, 1977) and detected by exposure to iodine vapour. The spots corresponding to PtdA were scraped into vials and counted for radioactivity in a liquid-scintillation counter. Note that under the conditions of these experiments changes in $[^{32}P]\text{PtdA}$ reflect changes in PtdA concentration (Holmsen *et al.*, 1984) and the DG precursor of PtdA most probably derives from hydrolysis of phosphoinositides (MacIntyre & Pollock, 1983).

Materials

Quin2 acetoxymethyl ester was purchased from Lancaster Synthesis, Morecambe, Lancs., U.K. Carrier-free $[^{32}P]P_i$ was supplied by the Regional Isotope Dispensary, Western Infirmary, Glasgow, Scotland, U.K. VP and PMA were obtained from Sigma and ionomycin was purchased from Calbiochem. PMA and ionomycin were dissolved in DMSO. All other reagents were of the highest purity available and were obtained as previously described (Pollock *et al.*, 1984).

RESULTS

In agreement with previous reports (Grant & Scrutton, 1980), aggregation and ATP secretion induced by submaximal concentration of VP ($3\text{--}30 \text{ nM}$) were augmented significantly by simultaneous or prior ($10\text{--}60 \text{ s}$) addition of sub-threshold concentrations of adrenaline ($0.1\text{--}1 \mu M$). The degree of potentiation observed apparently was independent of the time of exposure to adrenaline (Fig. 1).

With platelets suspended in medium of external free $[Ca^{2+}] = 1 \text{ mM}$, VP ($10 \text{ nM}\text{--}1 \mu M$), but not adrenaline ($< 5 \mu M$), induced concentration-dependent elevation of $[Ca^{2+}]_i$ from the basal value of around 90 nM to a maximum of $300\text{--}500 \text{ nM}$ in different experiments and with platelets from different donors. VP-induced elevation of $[Ca^{2+}]_i$ was decreased by around 80% by prior (60 s) addition of EGTA (4 mM) (results not shown). The residual signal observed in the presence of EGTA is attributed to mobilization of Ca^{2+} from internal stores (Hallam *et al.*, 1984; MacIntyre *et al.*, 1985). Addition of adrenaline ($0.1\text{--}5 \mu M$) to platelets suspended in medium of external free $[Ca^{2+}] = 1 \text{ mM}$ resulted in enhancement of VP-induced elevation of $[Ca^{2+}]_i$ (Fig. 2). However, unlike the aggregation and secretion responses, the degree of potentiation varied with the time at which adrenaline was added, and was significant only at early time points ($0\text{--}10 \text{ s}$). VP (10 nM)-induced mobilization of internal Ca^{2+} (i.e. monitored in the presence of 4 mM -EGTA) also was potentiated by $60 \pm 25\%$ (mean $\pm$ S.D., $n = 3$) after preincubation (10 s) with adrenaline (100 nM) (results not

Fig. 3. Effects of adrenaline on vasopressin-induced $[^{32}\text{P}]\text{PtdA}$ formation

Platelets were incubated with saline or adrenaline (ADR; $0.1 \mu\text{M}$) for the times indicated before addition of VP (30 nM) for a further 30 s. $[^{32}\text{P}]\text{PtdA}$ formation was monitored as described in the Methods section. Results are expressed as a percentage of the saline-treated control, which represents a 2–3-fold stimulation of $[^{32}\text{P}]\text{PtdA}$ formation. Incubations were carried out in the presence of the cyclo-oxygenase inhibitor flurbiprofen ($10 \mu\text{M}$). The results shown are means $\pm$ S.E.M. from a single experiment which was typical of three similar experiments using blood from different donors. Data were compared with the saline control by Student's *t* test: **P* < 0.05, ***P* < 0.005.

shown). It should be noted that, in all cases, the magnitude of the elevation of $[\text{Ca}^{2+}]_i$ was relatively small, even in the presence of adrenaline. In the presence of external Ca^{2+} , the rise elicited by VP (10 nM) alone ranged from 30 to 100 nM above basal, and these increased to 50–200 nM above basal in the presence of adrenaline.

To investigate the possible involvement of inositol lipid hydrolysis and DG formation in the potentiation of platelet activation, we examined the effects of adrenaline and VP on platelet $[^{32}\text{P}]\text{PtdA}$ formation. VP (10 nM– $1 \mu\text{M}$), but not adrenaline (< $10 \mu\text{M}$) elicited, in a concentration-dependent manner, formation of $[^{32}\text{P}]\text{PtdA}$, up to 3-fold above the basal value (results not shown; Pollock & MacIntyre, 1986). The stimulation of $[^{32}\text{P}]\text{PtdA}$ formation by sub-maximal concentrations of VP (10–100 nM) was augmented by adrenaline: significant potentiation was observed when adrenaline was added simultaneously with, or 10–60 s before, VP (Fig. 3).

To clarify the role of DG formation (generated as a consequence of inositol lipid metabolism) and elevation of $[\text{Ca}^{2+}]_i$ in the potentiation of platelet activation, we examined the effects of PMA (effectively a mimic of endogenous DG) and ionomycin (effectively a mimic of elevated $[\text{Ca}^{2+}]_i$) on VP-induced platelet aggregation and ATP secretion, using quin2-loaded platelets. Ionomycin at concentrations (10–100 nM) which produced small rises in $[\text{Ca}^{2+}]_i$ (Table 1) and which did not induce platelet activation (as monitored photometrically) when added to platelet suspensions for 10 s before addition of submaximal concentrations of VP resulted in enhancement of both the rate and the extent of VP-induced platelet aggregation. However, at these concentrations, ionomycin failed to convert the VP-induced reversible response ('primary' aggregation) into an irreversible response associated with ATP secretion ('secondary' aggregation) (Fig. 4). In contrast, sub-threshold concentrations of adrenaline markedly potentiated VP-induced

Table 1. Effects of ionomycin and vasopressin on platelet $[\text{Ca}^{2+}]_i$

Samples were incubated with VP and/or ionomycin, and $[\text{Ca}^{2+}]_i$ was monitored as described in the Methods section. DMSO (0.5%), the ionomycin vehicle, had no significant effect on VP-induced elevation of $[\text{Ca}^{2+}]_i$. The results shown are means $\pm$ S.E.M. of three experiments using blood from different donors.

[Ionomycin] (nM)	Increase in $[\text{Ca}^{2+}]_i$ (nM)	
	Without VP	With VP
0	—	70 ± 15
10	15 ± 3	80 ± 15
50	50 ± 10	180 ± 45
100	210 ± 45	300 ± 60

aggregation and ATP secretion, resulting in irreversible ('secondary') aggregation (Fig. 4). Note that, under these experimental conditions, the elevation of $[\text{Ca}^{2+}]_i$ induced by ionomycin in combination with VP was greater than or equivalent to that elicited by VP in combination with adrenaline (Table 1 and Fig. 2).

Preincubation of platelets with sub-threshold concentrations of PMA (< 10 nM; 30 s) resulted in a small but significant potentiation of VP-induced 'primary' aggregation, with no enhancement of ATP secretion (Fig. 4). Higher concentrations of PMA resulted in full aggregation and ATP secretion, when added alone (results not shown). Incubation of platelets with PMA (10 nM) and ionomycin (100 nM) in combination had little direct effect on platelet aggregation (Fig. 4a). Moreover, the extent of ATP secretion evoked by this agonist combination was $1 \pm 2\%$ (mean $\pm$ S.E.M., *n* = 5) of the maximum releasable ATP (defined as that elicited by 1 unit of thrombin/ml).

However, the subsequent addition of VP resulted in rapid irreversible aggregation accompanied by secretion of ATP, and the extent of the response was comparable with that elicited by adrenaline and VP in combination (Fig. 4).

DISCUSSION

Activation of human platelets by adrenaline is mediated via combination with α_2 -adrenoceptors and is not associated with inositol lipid hydrolysis or elevation of $[\text{Ca}^{2+}]_i$, as monitored by quin2 (MacIntyre *et al.*, 1985) but may be associated with N_i -mediated inhibition of adenylate cyclase (Aktories & Jakobs, 1985). In contrast, human platelet receptors for VP conform to the V_1 -receptor subtype, and receptor occupancy by agonists is coupled to inositol phospholipid hydrolysis and elevation of $[\text{Ca}^{2+}]_i$ (MacIntyre *et al.*, 1985; Pollock & MacIntyre, 1986; Siess *et al.*, 1986). Moreover, at least in intact platelets, agonist occupancy of V_1 receptors does not influence adenylate cyclase activity or cyclic AMP concentration (Vanderwel *et al.*, 1983). Adrenaline potentiates VP-induced platelet activation (Grant & Scrutton, 1980). Since VP and adrenaline apparently utilize distinct transduction processes to evoke platelet activation, such a combination would be most suitable to demonstrate synergism and to investigate the role, if any, of changes in phosphoinositide metabolism and elevation

Fig. 4. Effects of ionomycin and PMA on vasopressin-induced aggregation and ATP secretion

(a) Traces show typical aggregation responses from a single representative experiment. Samples were incubated with: DMSO (0.5%) plus VP; ionomycin (IONO; 100 nM) plus VP; PMA (10 nM) plus VP; PMA (10 nM) plus ionomycin (100 nM); or PMA (10 nM) plus ionomycin (100 nM) plus VP. The effect of adrenaline (ADR) preincubation is shown for comparison. (b) Results are means $\pm$ S.E.M. from five different experiments using blood from different donors: $\square$, rate of aggregation; $\blacksquare$, extent of ATP secretion. To facilitate comparison, results are shown as a percentage of the DMSO control in each experiment. DMSO (0.5%) had no significant effect on the vasopressin responses. The extent of ATP secretion evoked by vasopressin in the presence of DMSO was $15 \pm 1.5\%$ (mean $\pm$ S.E.M.) of the maximum releasable ATP. Data were compared with the DMSO control by Student's *t* test: **P* < 0.05, ***P* < 0.005.

of $[Ca^{2+}]_i$ in potentiation of platelet activation by adrenaline. The results of the present study demonstrate that the synergistic effects of adrenaline and VP in combination on platelet activation are accompanied by enhancement of VP-induced inositol lipid hydrolysis (monitored as $[^{32}P]$ PtdA formation) and elevation of $[Ca^{2+}]_i$ that derives partly from increased Ca^{2+} influx and partly from increased mobilization of intracellular Ca^{2+} . The apparent dissociation between the temporal effects of adrenaline on VP-induced phosphoinositide hydrolysis (Fig. 3) and elevation of $[Ca^{2+}]_i$ (Fig. 2) may merely reflect the greater resolution of the assay of $[^{32}P]$ PtdA, especially as the trend in $[Ca^{2+}]_i$ was always in the direction of an increase.

Even in the presence of adrenaline, agonist-induced

elevation of $[Ca^{2+}]_i$ only attains values around 150–300 nM (i.e. some 50–200 nM above basal). At these concentrations $[Ca^{2+}]_i$ itself, at least as monitored by quin2, is insufficient to account for the near-maximal stimulation of aggregation and ATP secretion observed under these circumstances (Rink *et al.*, 1982). This is confirmed by our observation that co-addition of VP and ionomycin, at concentrations which elevated $[Ca^{2+}]_i$ to an extent comparable with or greater than that evoked by VP and adrenaline in combination, resulted in only slight enhancement of aggregation, with no increment in ATP secretion. Thus potentiation of VP-induced platelet activation by adrenaline cannot be attributed to enhanced elevation of $[Ca^{2+}]_i$ alone.

PMA mimics the effect of endogenous DG in

activating protein kinase C and modulating platelet reactivity (Yamanishi *et al.*, 1983). PMA at sub-threshold concentrations resulted in only marginal enhancement of VP-induced platelet aggregation, without augmenting ATP secretion. Thus potentiation of VP-induced platelet activation by adrenaline cannot be mimicked by addition of an exogenous activator of protein kinase C and therefore may not be attributable to enhanced DG formation alone.

In contrast, the effect of adrenaline on VP-induced platelet aggregation and ATP secretion could be reproduced by sub-threshold concentrations of PMA and ionomycin in combination. Consequently, the mechanisms underlying potentiation of platelet activation by adrenaline may involve enhanced inositol lipid hydrolysis (and thus DG formation) and elevation of $[Ca^{2+}]_i$, perhaps consequent on formation of inositol 1,4,5-trisphosphate (Berridge, 1984).

These effects may be explained were adrenaline, either as a direct consequence of α_2 -adrenoceptor occupancy or as a consequence of decreased cyclic AMP concentration in a specific intracellular compartment, to induce a transient change in membrane lipid architecture (Irvine, 1982) or in a guanine-nucleotide-binding regulatory protein (Haslam & Davidson, 1984) and thus promote coupling of V_1 receptors to hydrolysis of phosphatidylinositol 4,5-bisphosphate via phospholipase C. Clearly, clarification of the roles of products of inositol phospholipid metabolism in the potentiation of platelet activation by adrenaline requires further investigation.

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