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Platelet aggregation induced by α_2-adrenoceptor and protein kinase C activation

A novel synergism

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Adrenaline or UK 14304 (a specific α_2-adrenoceptor agonist) and phorbol ester (phorbol 12,13-dibutyrate; PdBu) or bioactive diacylglycerols (*sn*-1,2-dioctanoylglycerol; DiC_8) synergistically induced platelet aggregation and ATP secretion. The effect on aggregation was more pronounced than the effect on secretion, and it was observed in aspirinized, platelet-rich plasma or suspensions of washed aspirinized platelets containing ADP scavengers. No prior shape change was found. In the presence of adrenaline, DiC_8 induced reversible aggregation and PdBu evoked irreversible aggregation that correlated with the different kinetics of DiC_8- and PdBu-induced protein kinase C activation. Adrenaline and UK 14304 did not induce or enhance phosphorylation induced by DiC_8 or PdBu of myosin light chain (20 kDa), the substrate of protein kinase C (47 kDa), or a 38 kDa protein. Immunoprecipitation studies using a $G_{common}\alpha$ antiserum or a $G_i\alpha$ antiserum showed that $G_i\alpha$ is not phosphorylated after exposure of platelets to PdBu or PdBu plus adrenaline. Adrenaline, PdBu or adrenaline plus PdBu did not cause stimulation of phospholipase C as reflected in production of [^{32}P]phosphatidic acid. Adrenaline caused a small increase of Ca^{2+} in the platelet cytosol of platelets loaded with Indo-1; this effect was also observed in the absence of extracellular Ca^{2+}. However, under conditions of maximal aggregation induced by adrenaline plus PdBu, no increase of cytosolic Ca^{2+} was observed. Platelet aggregation induced by PdBu plus adrenaline was not inhibited by a high intracellular concentration of the calcium chelator Quin-2. These experiments indicate that α_2-adrenoceptor agonists, known to interact with G_i, and protein kinase C activators synergistically induced platelet aggregation through a novel mechanism. The synergism occurs distally to G_i protein activation and protein kinase C-dependent protein phosphorylation and does not involve phospholipase C activation or Ca^{2+} mobilization.

INTRODUCTION

Adrenaline has direct effects on platelets that are not mediated by ADP, endoperoxides or thromboxane A_2 liberated from platelets [1–3]. It induces primary platelet aggregation [1–4], potentiation of stimulus-induced aggregation and secretion [4–8] and inhibition of adenylate cyclase through activation of α_2-adrenergic receptors [9,10]. α_2-Adrenoceptors in platelets are known to be coupled to the guanine-nucleotide-binding protein G_i, which mediates inhibition of adenylate cyclase [11,12]. Inhibition of adenylate cyclase is not, however, the mechanism by which adrenaline induces and potentiates aggregation because (*a*) in platelet-rich plasma, adrenaline-induced primary aggregation is associated with an increase rather than a decrease of cyclic AMP levels [13]; (*b*) adrenaline [4–8], but not a direct inhibitor of adenylate cyclase (2′,5′-dideoxyadenosine) [14], potentiates stimulus-induced aggregation and secretion; and (*c*) various classes of α_2-adrenoceptor agonists can be distinguished that either induce and potentiate platelet aggregation or inhibit adenylate cyclase, or do both [15,16].

Under conditions in which adrenaline induces primary aggregation, no activation of phospholipase C is observed [17]. In addition, adrenaline does not seem to activate protein kinase C [18] or to increase Ca^{2+} in the platelet cytosol [19,20]. Thus the effector mechanism by which α_2-adrenoceptor activation causes and potentiates platelet aggregation is unknown.

Phorbol esters and bioactive diacylglycerols share some properties of adrenaline: they do not induce the typical shape change response in the aggregometer [21], and do not activate phospholipase C [22,23] or increase Ca^{2+} in the platelet cytosol [24]. In contrast to α_2-adrenoceptor agonists, they do activate protein kinase C [25]. Although maximal activation of protein kinase C does not necessarily cause platelet aggregation and secretion, protein kinase C activation can synergize with Ca^{2+} mobilization to induce platelet responses [23,26]. On the other hand, protein kinase C can cause a desensitization of receptor-mediated platelet responses [27–30].

We recently observed that phorbol ester added to platelet-rich plasma sensitized platelets to activation by various physiological agonists and that the sensitizing effect of phorbol ester on platelet aggregation induced by adrenaline was unique among several platelet agonists studied [31]. The present study was developed to in-

Abbreviations used: PdBu, phorbol 12,13-dibutyrate; DiC_8, *sn*-1,2-dioctanoylglycerol; OAG, 1-oleoyl-2-acetylglycerol; G_i, GTP-binding protein which inhibits adenylate cyclase; PGI_2, prostacyclin.

vestigate the biochemical features underlying the synergistic interaction of α_2-adrenoceptor stimulation and protein kinase C activation. Evidence is presented for a novel synergism of protein kinase C with the signal transducing/effector mechanism coupled to α_2-adrenoceptor activation.

EXPERIMENTAL

Materials

Phorbol 12,13-dibutyrate (PdBu), ATP, GTP, thymidine, NADP, saponin, Protein A–Sepharose CL4B and 1-oleoyl-2-acetylglycerol (OAG) were purchased from Sigma (St. Louis, MO, U.S.A.). *sn*-1,2-Dioctanoylglycerol (DiC_8) was a gift from Dr. Robert M. Bell, Duke University (Durham, NC, U.S.A.). Purified G_i/G_o standard was kindly provided by Dr. Peter Gierschik (Heidelberg, Germany). Indo-1/AM and Quin-2/AM were from Calbiochem (La Jolla, CA, U.S.A.). Antiserum AS7 was generously provided by Dr. Allen Spiegel (NIH, Bethesda, MD, U.S.A.). Affinity-purified antiserum A569 was generously given by Dr. S. Mumby and Dr. A. Gilman (University of Texas Health Science Center, Dallas, TX, U.S.A.). UK 14304 was from Pfizer (Sandwich, Kent, U.K.). Pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.). Guanabenz was from Wyeth Laboratories (Philadelphia, PA, U.S.A.). All other materials were obtained as previously described [32–34]. [*adenylate*-^{32}P]NAD (800 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). The GTP-binding protein fragment of G_α against which AS569 was raised was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.).

Labelling of platelets with ^{32}P

Platelet-rich plasma (40–50 ml) was obtained from 110 ml of human blood anticoagulated with $\frac{1}{10}$ vol. of 3.8% (w/v) trisodium citrate. Prostacyclin (PGI_2) (20 ng/ml) was added, and platelets were pelleted by centrifugation for 10 min at 800 ***g***. Platelets were resuspended in 2 ml of a phosphate-free buffer (pH 7.35) containing NaCl (138 mM), KCl (2.9 mM), Hepes (20 mM), $MgCl_2,6H_2O$ (1 mM), EGTA (1 mM) and glucose (5 mM). Aspirin (1 mM), apyrase (0.8 units of ADPase/ml) and PGI_2 (500 ng/ml) were added, and platelets were incubated with 4–5 mCi of ^{32}P at 37 °C for 90 min. In other experiments, labelling of platelets with ^{32}P was performed as described [31,33], with essentially similar results obtained. At the end of the incubation, 10 ml of prewarmed (37 °C) buffer containing apyrase and PGI_2 was added, and platelets were pelleted by centrifugation for 10 min at 800 ***g***. Platelets were resuspended in 10 ml of the buffer (37 °C) as described above but containing $CaCl_2$ (0.1 mM) instead of EGTA. Apyrase (0.6 units of ADPase/ml) or, in experiments in which ATP secretion was measured, phosphocreatine (2 mM) and creatine kinase (20 units/ml) were added to inactivate ADP in the extracellular medium.

Samples (0.6 ml) were transferred into aggregometer cuvettes. PdBu (stock solutions 100 μM and 10 μM in dimethyl sulphoxide), DiC_8 (stock solution 1 mM in ethanol) or OAG (stock solution 50 mg/ml in ethanol) was added simultaneously with or 5–10 s before *l*-adrenaline or UK 14304. Aggregation was measured in a Chronolog aggregometer adjusted for maximal sensitivity (increased light transmission is expressed in mm of pen deflection). Aggregation and concomitant ATP secretion were measured in a Lumi-Aggregometer using the Luciferin/Luciferase reagent (Chronolog Inc., Havertown, PA, U.S.A.). For better sensitivity of the aggregation measurement, the platelet suspension was calibrated against a platelet suspension diluted 1:4 to 1:3 with buffer.

Samples were incubated at 37 °C whilst stirring. Aliquots (0.05 ml) were transferred into an equal volume of sample buffer containing 1% SDS, dithiothreitol (15 mg/ml) and glycerol (1%) for measurement of protein phosphorylation, or into 0.375 ml of chloroform/methanol (1:2, v/v) for measurement of [^{32}P]phosphatidic acid [30]. ^{32}P-Labelled proteins were separated on SDS/polyacrylamide (11%) gels using a Tris buffer with a pH of 8.8. Specific zones of the dried gels localized by autoradiography were cut out and measured for ^{32}P radioactivity by liquid scintillation counting [32]. The experiments shown are representative of at least four others.

Pertussis toxin-dependent ADP-ribosylation of platelet G_i

Platelet-rich plasma (40–50 ml) from 110 ml of blood was incubated with 1 mM-aspirin for 15 min at 37 °C and, after addition of PGI_2 (50 ng/ml), was centrifuged. The platelet pellet was washed in 20 ml of buffer containing 1 mM-EGTA and apyrase (0.6 units of ADPase/ml), centrifuged (after addition of PGI_2) and resuspended in 10 ml of buffer without apyrase or EGTA. Samples (1.3 ml) of platelet suspension were incubated for 2 min at 37 °C without stirring in aggregometer cuvettes under continuous recording of light transmission. Aliquots (0.2 ml) of platelet suspensions before (controls) and at various times after exposure to adrenaline, PdBu or a combination of both were transferred to 0.05 ml of the ADP-ribosylation mixture.

The ADP-ribosylation mixture was freshly prepared and contained (final concentrations in the platelet suspension) EDTA (1 mM), pertussis toxin (7 μg/ml), NAD (100 μM), [^{32}P]NAD (30 μCi/ml), NADP (1 mM), GTP (100 μM), saponin (100 μg/ml), thymidine (10 mM) and ATP (1 mM) (see also ref. [35]). The pertussis toxin was preactivated in 1 mM-dithiothreitol at 37 °C for 30 min. Pertussis toxin-dependent ADP-ribosylation of the platelet lysate was carried out for 1 h at 37 °C. After addition of EDTA (10 mM), the platelet membranes were pelleted by centrifugation (13000 ***g*** for 5 min) and resuspended in 0.1 ml of sample buffer. ^{32}P-labelled ADP-ribosylated $G_i\alpha$ subunits were separated on SDS/polyacrylamide (11%) gels and quantified as described above. The experiment shown is representative of ten others.

Immunoprecipitation of $G_i\alpha$ from platelets

Immunoprecipitation of $G_i\alpha$ was carried out according to the methods described in refs. [36] and [37]. Two antisera were used: the specific antiserum (AS7), which was produced in rabbits against the *C*-terminal decapeptide of rod transducin, and recognizes $G_i\alpha$ subunits, and the antiserum A569, which was produced in rabbits against the pentadecapeptide GAGESGKSTIVKQMK that is located near the *N*-terminal region and recognizes all G_α subunits.

^{32}P-Labelled ADP-ribosylated platelet membranes dissolved in water (0.2 ml) or ^{32}P-prelabelled platelet suspensions (0.2 ml; exposed to adrenaline, PdBu or both) were solubilized with 0.2 ml of buffer (pH 7.2) containing 1% SDS, aprotinin (20 μg/ml), 0.4 mM-$NaVO_4$, 2 mM-dithiothreitol, 150 mM-NaCl and 50 mM-Na_2HPO_4, and then they were heated for 1 min at 90 °C. Then, 40 μl of 10 × RIPA (10% deoxycholate, 10% Triton X-100, 150 mM-NaCl and 50 mM-Na_2HPO_4) was added to the clear extract and incubated with 4 μl of normal rabbit serum ('control') for 45 min on ice. Protein A–Sepharose (12 mg in 150 μl of RIPA) or Protein A (extracellular)–Agarose (1 mg in 100 μl of RIPA) was added and incubated for 30 min on ice. After centrifugation in a microfuge for 5 min, the supernatant (550 μl) was incubated at 4 °C with 3 μl of antiserum AS7 or 4 μl of affinity-purified A569 for 2 h, and then with Protein A–Sepharose or Protein A–Agarose for 1 h. After centrifugation, the supernatant was discarded, and Protein A–Sepharose or Protein A–Agarose of both control and antiserum-incubated samples was washed four times with 1 ml of RIPA buffer and once with phosphate-buffered saline. The samples were resuspended in 100 μl of sample buffer and heated for 3 min at 100 °C, and the supernatant was separated by SDS/polyacrylamide (11%)-gel electrophoresis. Proteins were stained with silver nitrate and dried gels were exposed to X-OMAT films at −80 °C (during 2–5 days) for autoradiography. The experiments shown are representative of four others.

Measurement of Ca^{2+} mobilization

Platelet-rich plasma from 160 ml of freshly drawn blood anticoagulated with trisodium citrate (0.38%, w/v) was incubated with 5 μM-Indo-1/AM (dissolved in dimethyl sulphoxide) and 1 μM-prostaglandin E_1 for 45 min at 37 °C. Then aspirin (1 mM) was added and incubation was continued for a further 15 min. Platelets were pelleted by centrifugation and resuspended in 30 ml of buffer containing apyrase (0.6 units of ADPase/ml) and 0.01 mM-$CaCl_2$. Ca^{2+} measurements were performed in 1 ml aliquots of platelet suspension which had been prewarmed to 37 °C for 2 min. Fluorescence (F) was recorded using a Perkin–Elmer 512 double-beam fluorescence spectrophotometer with the emission wavelength set to 390 nm and the excitation wavelength set to 340 nm. F, $F_{max.}$, $F_{min.}$ and F_{leak} were recorded, and the intracellular Ca^{2+} concentrations were calculated as described in refs. [38] and [39]. The experiments shown are representative of five others.

RESULTS

Synergism of α_2-adrenoceptor agonists and phorbol ester in inducing platelet aggregation and secretion

Adrenaline added before, with or after PdBu profoundly potentiated the rate and extent of aggregation of washed platelets (Fig. 1) and platelet-rich plasma [31]. This synergism was independent of the formation of endoperoxides/thromboxane A_2 or the release of ADP, since it was observed in aspirinized platelets containing ADP scavengers (phosphocreatine/creatine kinase or apyrase). In the presence of adrenaline, DiC_8 induced a reversible aggregation response, whereas aggregation induced by PdBu was irreversible (Fig. 1). These effects closely correlate with the differential effects of DiC_8 and

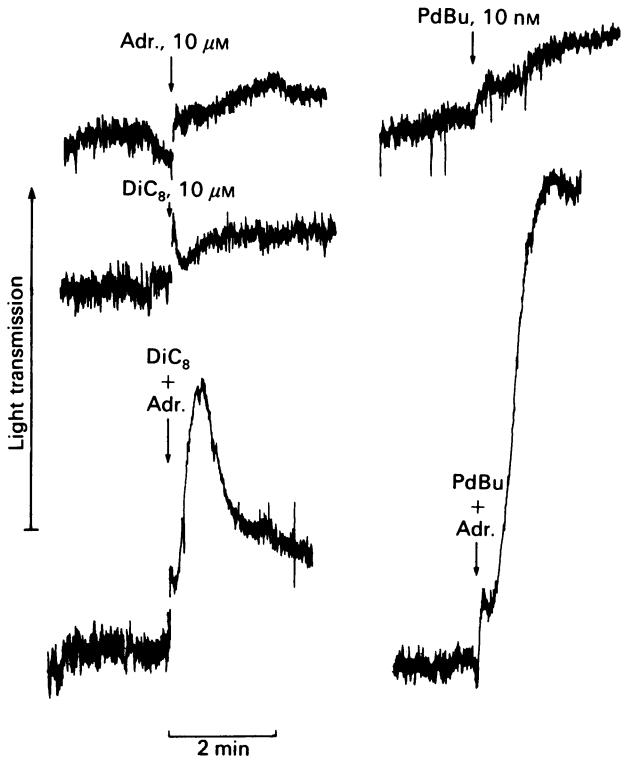

Fig. 1. Synergism of adrenaline plus DiC_8 or PdBu on platelet aggregation

Aspirinized, ^{32}P-prelabelled, washed platelets were resuspended in buffer containing apyrase (0.6 units of ADPase/ml) and samples (0.6 ml) were exposed to DiC_8 (added in ethanol, final concentration 1%) or PdBu with or without adrenaline (Adr.). Aggregation was measured using a single channel aggregometer adjusted to maximal sensitivity. Note that the initial upward deflection of the pen after addition of the agonists is due to dilution of the platelet suspension with the solvent of the agonist solution.

PdBu on protein phosphorylation in platelets. Protein kinase C activation induced by DiC_8 was reversible; protein kinase C activation evoked by PdBu was irreversible (Figs. 2 and 3).

The synergistic effect of PdBu plus adrenaline on platelet aggregation was dependent on the concentration of both PdBu and adrenaline; 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) was similarly effective, whereas the inactive parent compound 4α-phorbol was ineffective [31]. The preincubation time of platelets with PdBu did not affect its synergism with adrenaline on platelet aggregation; it could be extended up to 12 min without observing a decrease of the potentiation of aggregation [31]. Platelets were preincubated up to 3 min with adrenaline before addition of PdBu, and the synergistic action of both agonists on platelet aggregation was not decreased (results not shown). The action of adrenaline was imitated by the synthetic α_2-adrenoceptor agonist UK 14304 (Table 1).

The synergism of PdBu and adrenaline on ATP secretion was quantitatively much less pronounced and only observed at higher phorbol ester concentrations (Table 1). Secretion of ATP started later than aggregation. PdBu, adrenaline or the simultaneous addition of both agonists did not induce shape change (Fig. 1).

Table 1. Effects of UK 14304, a specific α_2-adrenoceptor agonist, and PdBu on platelets

Formation of [^{32}P]phosphatidic acid was measured 1 min after addition of the stimuli; protein phosphorylation and platelet responses were measured 2 min afterwards. Values indicate means ± S.D. for triplicate incubations or samples. The experiment is representative for two others.

	Aggregation (mm per deflection)	ATP secretion (μM)	Phosphatidic acid (c.p.m.)	Protein phosphorylation (c.p.m. of ^{32}P)	
				47 kDa	20 Da
Control	0	0	2853 ± 328	745 ± 98	400 ± 35
PdBu, 10 nM	16	0	2697	1346 ± 2	434 ± 17
PdBu, 20 nM	26	2.5	2595	1888 ± 84	480 ± 32
Adrenaline, 20 μM	12	0	2995	763 ± 12	410 ± 2
Adrenaline, 20 μM + PdBu, 10 nM	46	0	3108	1257 ± 45	433 ± 20
Adrenaline, 20 μM + PdBu, 20 nM	112	4.0	2362	1908 ± 106	523 ± 22
UK 14304, 20 μM	4	0	2979	728 ± 25	414 ± 25
UK 14304, 20 μM + PdBu, 10 nM	42	0	2734	1391 ± 51	421 ± 9
UK 14304, 20 μM + PdBu, 20 nM	82	3.5	2403	1762 ± 18	475 ± 36

α_2-Adrenoceptor activation does not alter protein phosphorylation induced by DiC_8 or PdBu

DiC_8 and PdBu evoked the phosphorylation of several proteins in washed, aspirinized, ^{32}P-labelled platelets (Fig. 2; similar results obtained with PdBu are now shown). The increase of phosphorylation has been quantified for the following ^{32}P-labelled proteins: a 20 kDa protein (myosin light chain), a 38 kDa protein of unknown function, and the 47 kDa protein that is the substrate for protein kinase C. Under conditions in which DiC_8 (10 μM) or PdBu (40 nM) induce maximal phosphorylation of the 47 kDa protein (Figs. 2 and 3), no significant effects on platelet aggregation were observed. Low concentrations of PdBu (5–10 nM) in the absence of plasma, or high concentrations of PdBu (40 nM) in the presence of 5% plasma, induced phosphorylation of the 47 kDa protein, but not the 20 kDa or 38 kDa proteins (Fig. 3; Table 1).

Individually, adrenaline and UK 14304 did not stimulate protein phosphorylation. Neither adrenaline nor UK 14304 significantly enhanced protein phosphorylation induced by DiC_8 or PdBu (Figs. 2 and 3). Also, protein phosphorylation induced by submaximal concentrations of PdBu was not enhanced by adrenaline or UK 14304 (Table 1).

Immunoprecipitation of $G_i\alpha$

We observed that PdBu and DiC_8 enhanced the phosphorylation of an approx. 38–39 kDa protein in whole platelets (Figs. 2 and 3). It was further found that

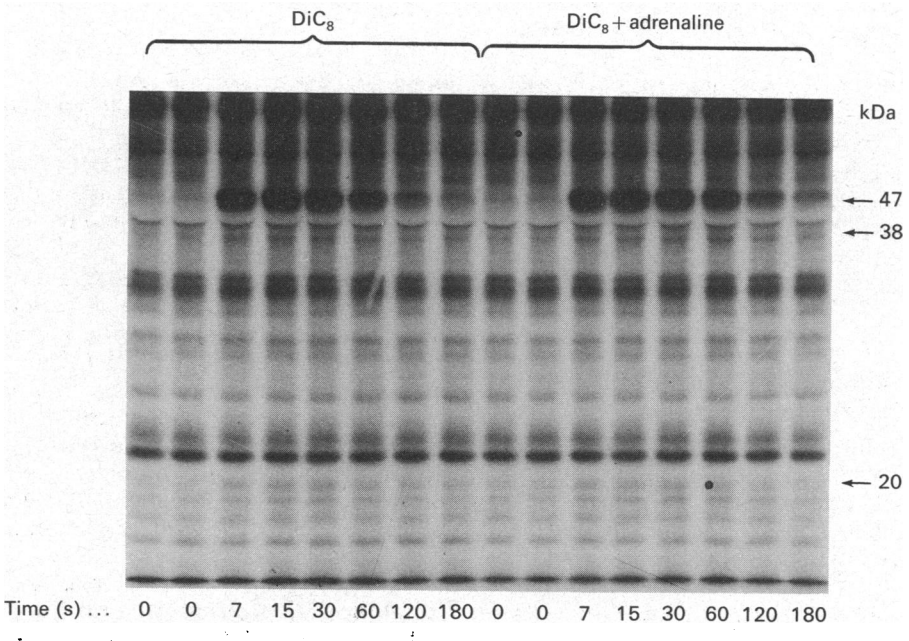

Fig. 2. Time courses of protein phosphorylation induced by DiC_8 or by DiC_8 plus adrenaline in human platelets

The Figure shows an autoradiograph of [^{32}P]phosphorylated platelet proteins. Experimental conditions were the same as described in legend to Fig. 1.

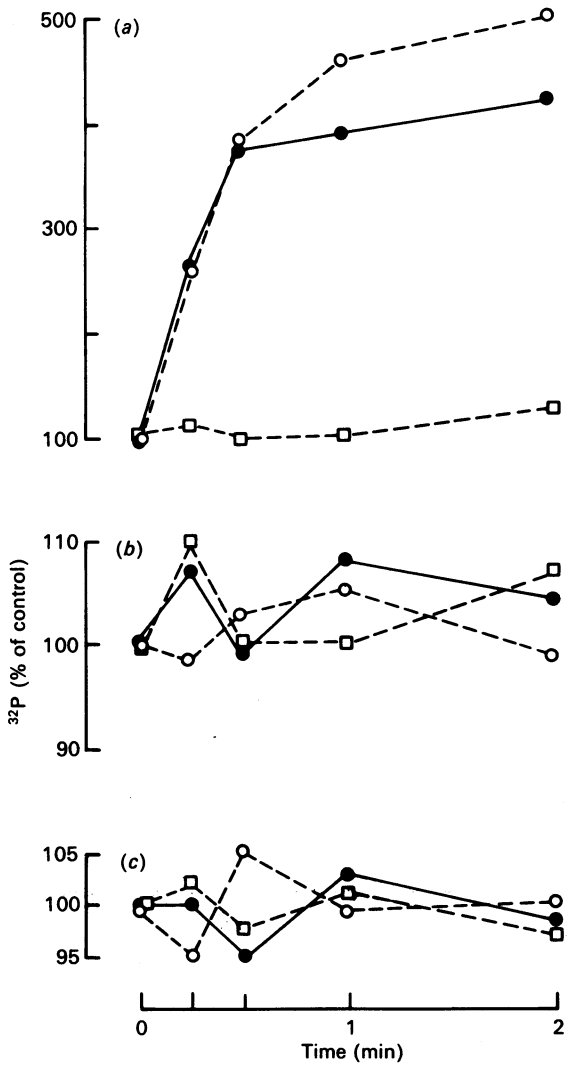

Fig. 3. Time course of protein phosphorylation and phosphatidic acid formation in platelets exposed to adrenaline (10 μM) (□), PdBu (40 nM) (○), or adrenaline plus PdBu (●)

(*a*) 47 kDa protein, (*b*) 20 kDa protein, (*c*) phosphatidic acid. Experimental conditions were as in Fig. 1, and the resuspension buffer also contained EGTA (4 mM), platelet-poor plasma (5%) and hirudin (1 unit/ml). Platelet aggregation measured as % light transmission was: PdBu, 0; adrenaline, 4; adrenaline plus PdBu, 37. The experiment is representative of three others.

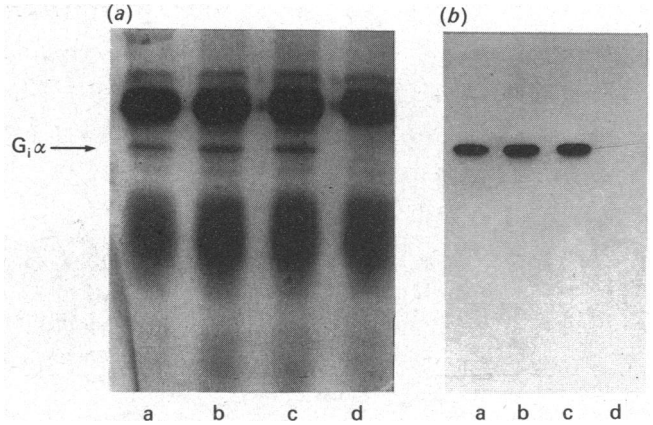

Fig. 4. Immunoprecipitation of ADP-ribosylated $G_i\alpha$ from platelet membranes

Various amounts of [^{32}P]ADP-ribosylated platelet membranes (see the Experimental section) were incubated with rabbit antiserum AS7 (lanes a, b and c) or control serum (lane d) and precipitated using Protein A–Sepharose. (*a*) Silver stain of the immunoprecipitate after SDS/gel electrophoresis, (*b*) autoradiograph. Lane a, 0.25 mg; b, 0.5 mg; c, 1 mg; d, 1 mg of platelet membrane protein. In lane a, recovery of the added ADP-ribosylated platelet membrane $G_i\alpha$ was 15% after immunoprecipitation.

this protein was present in platelet membranes and comigrated with the $G_i\alpha$ from bovine brain as well as the ^{32}P-labelled, ADP-ribosylated $G_i\alpha$ from platelet membranes (results not shown). Since it has been reported that protein kinase C phosphorylates $G_i\alpha$ in the absence of β/γ subunits [40], we investigated whether the observed synergism of adrenaline and PdBu correlated with an increase of $G_i\alpha$ phosphorylation. We therefore performed immunoprecipitation studies using a $G_i\alpha$ antiserum (AS7) that specifically immunoprecipitated ^{32}P-labelled, ADP-ribosylated $G_i\alpha$ from platelet membranes (Fig. 4). This platelet $G_i\alpha$ was visible in silver-stained SDS/polyacrylamide gels and was detected by autoradiography. Incubation of platelet membranes with control rabbit serum did not immunoprecipitate ADP-ribosylated $G_i\alpha$ (Fig. 4). Further experiments were carried out to immunoprecipitate $G_i\alpha$ after exposure of ^{32}P-labelled intact platelets to adrenaline, PdBu or a combination of the two. It seemed that PdBu (50 nM) enhanced the ^{32}P-labelling of $G_i\alpha$, but adrenaline did not increase further the protein kinase C-dependent phosphorylation of $G_i\alpha$ (results not shown). Since several phosphorylated proteins were found after gel separation of the immunoprecipitates, more rigorously controlled experiments were performed. Using the $G_i\alpha$-antiserum AS7 or the $G_{common}\alpha$-antiserum A569, $G_i\alpha$ from platelets was specifically immunoprecipitated as seen by silver staining of the immunoprecipitates after gel separation (Figs. 4 and 5). Phorbol ester stimulation of platelets increased the phosphorylation of a protein that was present in the immunoprecipitates and comigrated with $G_i\alpha$ but was not $G_i\alpha$. The phosphorylated protein was also present under conditions in which platelet $G_i\alpha$ was not immunoprecipitated (Fig. 5, and results not shown). It seems, therefore, that protein kinase C does not phosphorylate $G_i\alpha$ in intact platelets.

Effects of adrenaline and PdBu on pertussis toxin-dependent ADP ribosylation of G_i

The amount of intact G_i in platelets can be measured by pertussis toxin-catalysed ADP ribosylation [41]. Exposure of platelets to adrenaline, known to induce dissociation of G_i in platelet membranes [11,12], did not reduce the subsequent pertussis toxin-induced ADP-ribosylation of G_i in saponized platelets. Also, PdBu and PdBu plus adrenaline had no effect (Table 2). In contrast, platelets treated with thrombin (0.1 unit/ml) showed a 50% decrease of pertussis-toxin-dependent ADP-ribosylation of G_i.

To support the hypothesis that the potentiating effect of adrenaline on platelet aggregation is mediated through

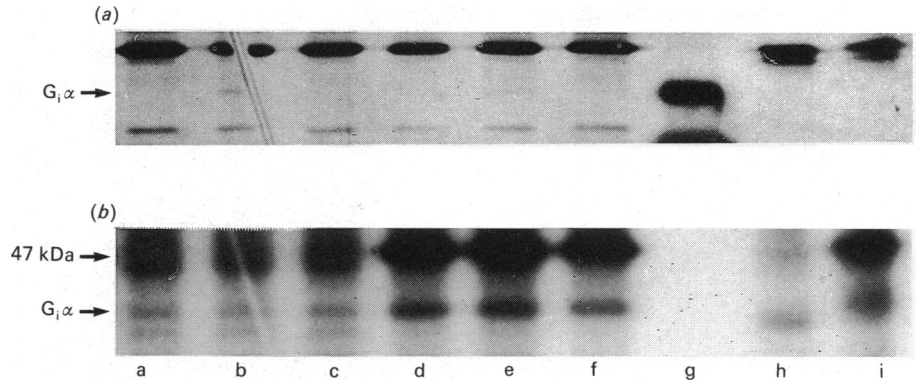

Fig. 5. Immunoprecipitation of $G_i\alpha$ by $G_{common}\alpha$ antiserum A569

Suspensions of washed platelets prelabelled with ^{32}P were incubated at 37 °C for 2 min with saline (lanes a, b, c and h) or PdBu, 200 nM (lanes d, e, f and i). Aliquots (0.2 ml) were transferred into 0.2 ml of lysis buffer and incubated with control serum as described in the Experimental section. After precipitation of the samples incubated with control serum, the supernatant was incubated with 4 μl of saline (lanes a and d), 4 μl of affinity-purified G_α-antiserum A569 (lanes b and e) or 4 μl of affinity-purified G_α-antiserum that had been preincubated with 20 μg of the specific G_α-protein fragment for 30 min at 37 °C (lanes c and f). The Figure shows the silver stain (*a*) and the corresponding autoradiograph (*b*) of the immunoprecipitates applied to a SDS/(10%) polyacrylamide gel. Lane g, G_i/G_o purified from bovine brain; lanes h and i, whole platelet suspensions (1 μl). The experiment is representative of two others.

G_i, we used guanabenz, which is a weak α_2-adrenoceptor agonist for the aggregatory response but does not cause inhibition of adenylate cyclase [15,16]. Therefore, guanabenz does not interact with $G_i\alpha$ [11]. Guanabenz (10–100 μM) added with PdBu or OAG to aspirinized platelets did not show synergistic action on platelet aggregation (results not shown).

Absence of phospholipase C activation and Ca^{2+} mobilization under conditions of maximal aggregation induced by adrenaline plus PdBu

The synergistic action of PdBu plus adrenaline on platelet aggregation was evident under conditions of virtual absence of extracellular Ca^{2+} (< 100 nM). Chelation of extracellular Ca^{2+} by EGTA (4 mM) decreased but did not eliminate platelet aggregation induced by PdBu plus adrenaline. Chelation of extracellular Ca^{2+} did not affect the action of PdBu or PdBu plus adrenaline on protein phosphorylation (results not shown).

We observed that adrenaline (2–10 μM) induced a small but significant increase of cytosolic Ca^{2+} from 86 ± 7 to 104 ± 6 nM (mean $\pm$ S.D., $n = 7$ experiments) in aspirinized, washed human platelets in the presence of apyrase and EGTA (2 mM). However, prior incubation of platelets with PdBu eliminated that small increase of cytosolic Ca^{2+} induced by adrenaline (Fig. 6). Maximal platelet aggregation observed after simultaneous addition of adrenaline plus PdBu was not accompanied by detectable Ca^{2+} mobilization. In contrast, a low concentration of vasopressin (20 mM), which only induced shape change, clearly enhanced Ca^{2+} in the platelet cytosol.

Adrenaline, PdBu or simultaneous addition to platelets of high concentrations of PdBu (40 nM) and adrenaline (20 μM) or UK 14304 (20 μM) that resulted in a maximal aggregation response did not cause an increase of [^{32}P]phosphatidic acid (Fig. 3; Table 1; results not shown). Therefore, maximal platelet aggregation induced by adrenaline plus PdBu could occur without phospholipase C activation and Ca^{2+} mobilization.

Loading platelets with high concentrations of Quin-2 (4–5 mM) that created a Ca^{2+} sink in the platelet cytosol and delayed vasopressin (100 nM)- and ionophore A23187 (15 μM)-induced platelet shape change and aggregation of platelet-rich plasma did not inhibit aggregation induced by PdBu or PdBu plus adrenaline (results not

Table 2. Effects of adrenaline and PdBu on pertussis toxin-dependent ADP-ribosylation of G_i

Intact platelets were exposed for the times indicated to adrenaline, PdBu, or both, and pertussis toxin-dependent ADP-ribosylation was measured after platelet lysis with saponin as detailed in the Experimental section.

Time after addition of stimulus (s)...	ADP-ribosylation (c.p.m. of ^{32}P)			
	0	10	20	60
Adrenaline, 20 μM	1025 ± 20	1068	1010	998
PdBu, 50 nM	1045 ± 38	1050	1084	1047
Adrenaline + PdBu	1060 ± 42	982	1030	964
α-Thrombin, 20 nM	1030 ± 32	851	460	348

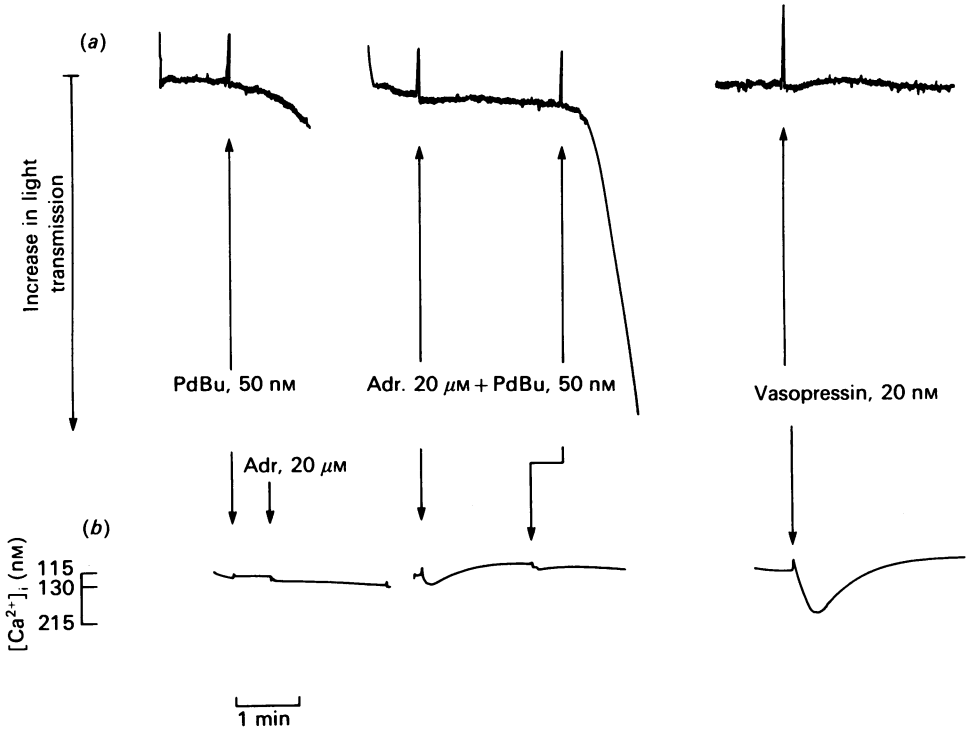

Fig. 6. Effects of PdBu, adrenaline or both on cytosolic Ca^{2+}

Aspirinized platelets loaded with Indo-1/AM resuspended in buffer containing $CaCl_2$ (0.01 mM) and apyrase were exposed to adrenaline (Adr.) (20 μM), PdBu (50 nM) or both at 37 °C. For comparison, vasopressin (20 nM) was added. (*a*) Aggregation tracings of parallel samples recorded in a Chronolog-aggregometer; (*b*) fluorescence tracings of Ca^{2+} monitored in a fluorimeter.

shown). These data also support the observation that increased cytosolic Ca^{2+} is not involved in the synergistic action of adrenaline plus PdBu.

DISCUSSION

Our study shows that α_2-adrenoceptor agonists and protein kinase C activators synergistically induce platelet aggregation and secretion. The mechanism underlying this synergism involves a novel interaction of two different signal-transducing pathways: protein kinase C and, probably, the GTP-binding protein G_i linked to α_2-adrenoceptors. Although protein kinase C activation by itself does not cause platelet aggregation, the activation of protein kinase C parallels platelet aggregation in the presence of adrenaline. Since α_2-adrenoceptor activation did not change the pattern and extent of protein phosphorylation induced by DiC_8 or PdBu, the mechanism responsible for that synergism must be found at a step distal to protein kinase C activation.

Adrenaline does not activate phospholipase C, as determined by the formation of 1,2-diacylglycerol [17], phosphatidic acid ([17]; Fig. 3) or inositol phosphates (results not shown) in aspirinized platelets containing ADP scavengers. Also, neither adrenaline nor the synthetic α_2-adrenoceptor agonist UK 14304 induced any protein phosphorylation. We observed that adrenaline induced a small cytosolic Ca^{2+} increase in human platelets. This increase, which is barely detectable, was observed in the presence of extracellular EGTA and reflects Ca^{2+} mobilization, probably from plasma membrane Ca^{2+} pools. Interestingly, Ca^{2+} mobilization in the absence of phospholipase C activation induced by α_2-adrenoreceptor activation has been reported recently in human erythroleukaemia cells [42].

Adrenaline neither increased the binding of PdBu to platelets [43] nor potentiated PdBu-induced protein kinase C activation. This may be explained by the failure of adrenaline to increase cytosolic Ca^{2+} to a significant level and to produce 1,2-diacylglycerol; both molecules are involved in the translocation of protein kinase C to the membrane [44]. Thus, α_2-adrenoceptor agonists synergize with protein kinase C by a mechanism that is clearly different from that observed with other platelet stimuli such as vasopressin, ADP, platelet-activating factor and prostaglandin endoperoxide analogues, which activate phospholipase C, increase Ca^{2+} in the cytosol and enhance PdBu binding to platelets [43]. These stimuli potentiate phorbol ester-induced protein kinase C activation, whereas adrenaline shows true synergism with protein kinase C at a step distal to the enzyme.

Phorbol esters and bioactive diacylglycerols have some properties in common with adrenaline: they do not induce the typical shape change from discoid to spheroid platelets; they evoke a small aggregation response; and they do not induce phospholipase C activation or an increase of Ca^{2+} in the platelet cytosol [19–24]. Our study shows that the addition of adrenaline plus PdBu does not induce phospholipase C activation or Ca^{2+} mobilization, although the extent of platelet aggregation is maximal. Furthermore, platelet aggregation induced by adrenaline and PdBu is not affected by high concentrations of intracellular Quin-2. Therefore, platelet aggregation can occur by a mechanism that is independent of

inositol trisphosphate formation and intracellular Ca^{2+} mobilization.

The most proximal biochemical event in the chain of the signal-transducing mechanism triggered by α_2-adrenoceptor activation is the dissociation of $G_i\alpha$ from β/γ of G_i [11,12]. The amount of intact (non-dissociated) G_i can be measured by pertussis toxin-dependent ADP-ribosylation [41,45]. Treatment of intact platelets with thrombin or collagen reduces the subsequent pertussis toxin-induced ADP-ribosylation of G_i proteins in saponized platelets [41]. These results had been interpreted as dissociation of G_i caused by receptor activation [41]. However, we found that adrenaline, in contrast to thrombin, did not reduce pertussis toxin-dependent ADP ribosylation of G_i. This might be explained by a 20- to 100-fold excess of G_i proteins over α_2-adrenergic receptors in platelets [45]. α_2-Adrenergic receptors may be coupled to fewer G_i molecules than thrombin receptors or, alternatively, the thrombin-induced decrease of pertussis toxin-dependent ADP-ribosylation of G_i may be caused by factors other than receptor-induced dissociation of G_i. In support for the latter, we observed that the Ca^{2+} ionophore A23187 also reduced pertussis toxin-dependent ADP-ribosylation of G_i proteins in platelets (W. Siess & E. G. Lapetina, unpublished work). Our results, showing functional synergism with the full α_2-adrenoceptor agonist UK 14304 but no synergism with the partial α_2-agonist guanabenz, which does not inhibit adenylate cyclase, indicate that G_i dissociation might be involved. Inhibition of adenylate cyclase is certainly not the mechanism by which adrenaline synergizes with phorbol esters in inducing platelet aggregation (see Introduction). In support of this fact, phorbol esters do not potentiate but rather attenuate the adrenaline-induced decrease of cyclic AMP levels in platelets exposed to prostaglandin E_1 [46].

$G_i\alpha$ could possibly produce actions in platelets other than inhibition of adenylate cyclase. The possibility that $G_i\alpha$ released from β/γ after adrenaline could be phosphorylated by protein kinase C [40] was investigated in the present study. We found that exposure of intact platelets to phorbol ester alone increased the phosphorylation of a protein that comigrated with $G_i\alpha$ on SDS gels and was also present in immunoprecipitates obtained after incubating platelet lysates with antiserum against $G_i\alpha$ or $G_{common}\alpha$. However, our results also show that this protein, which is phosphorylated by protein kinase C, is not identical to $G_i\alpha$. It seems that this phosphorylated protein, as well as other phosphorylated platelet proteins, binds directly to the Protein A–Sepharose or Protein A–Agarose that was used to precipitate the immunocomplexes.

Our study shows that the target of the synergism of adrenaline plus phorbol ester must be distal to G_i and protein kinase C. Although the messenger system of α_2-adrenoceptor action in platelets is unknown, one could suggest that it involves an ion channel corresponding to recent studies that link G_i activation to K^+ channels in cardiac tissue [47,48]. Also, α_2-adrenergic receptors in neuronal tissues seem to be linked via a pertussis toxin-sensitive G_i protein to K^+ channels [49]. On might then further speculate that such an ion channel activated by G_i might be modified by protein kinase C-dependent phosphorylation. The novel synergistic interaction shown in this study might apply to other agonists that, in different tissues, interact with G_i and activate protein kinase C.

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