Platelet aggregation induced by \( \alpha_2 \)-adrenoceptor and protein kinase C activation

A novel synergism

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Adrenaline or UK 14304 (a specific \( \alpha_2 \)-adrenoceptor agonist) and phorbol ester (phorbol 12,13-dibutyrate; PdBu) or bioactive diacylglycerols (sn-1,2-dioctanoylglycerol; DiC\(_4\)) 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\(_4\) induced reversible aggregation and PdBu evoked irreversible aggregation that correlated with the different kinetics of DiC\(_4\)- and PdBu-induced protein kinase C activation. Adrenaline and UK 14304 did not induce or enhance phosphorylation induced by DiC\(_4\) 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\(_{i}\) antiserum showed that G\(_{i}\) 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 \([^{32}\text{P}]\)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 \( \alpha_2 \)-adrenoceptor agonists, known to interact with Gi, and protein kinase C activators synergistically induced platelet aggregation through a novel mechanism. The synergism occurs distally to G, 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 \( \text{A}_2 \) liberated from platelets [1–3]. It induces primary platelet aggregation [1–4], potentiates of stimulus-induced aggregation and secretion [4–8] and inhibition of adenylyl cyclase through activation of \( \alpha_2 \)-adrenergic receptors [9,10]. \( \alpha_2 \)-Adrenoceptors in platelets are known to be coupled to the guanine-nucleotide-binding protein G\(_i\), which mediates inhibition of adenylyl cyclase [11,12]. Inhibition of adenylyl 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 adenylyl cyclase (2',5'-dideoxyadenosine) [14], potentiates stimulus-induced aggregation and secretion; and (c) various classes of \( \alpha_2 \)-adrenoceptor agonists can be distinguished that either induce and potentiate platelet aggregation or inhibit adenylyl 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 \( \alpha_2 \)-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 \( \alpha_2 \)-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-

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Abbreviations used: PdBu, phorbol 12,13-dibutyrate; DiC\(_4\), sn-1,2-dioctanoylglycerol; OAG, 1-oleoyl-2-acetylglycerol; G\(_i\), GTP-binding protein which inhibits adenylylate cyclase; PG\(_H_2\), prostacyclin.
vestigate the biochemical features underlying the synergistic interaction of \( \alpha_2 \)-adrenoceptor stimulation and protein kinase C activation. Evidence is presented for a novel synergism of protein kinase C with the signal transducing/effecter mechanism coupled to \( \alpha_2 \)-adrenoceptor activation.

**EXPERIMENTAL**

**Materials**

Phorbol 12,13-dibutyrate (PdBu), ATP, GTP, thymidine, NADP, saponin, Protein A-Sepharose CL4B and 1-oleoyl-2-acetyl-glycerol (OAG) were purchased from Sigma (St. Louis, MO, U.S.A.). sn-1,2-Dioctanoyl-glycerol (DiC\(_4\)) was a gift from Dr. Robert M. Bell, Duke University (Durham, NC, U.S.A.). Purified G\(_{15}/G\(_{21}\) standard was kindly provided by Dr. Peter Gierschik (Heidelberg, Germany). Indo-I/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. Mumbry 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\(_\alpha\) 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 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), Heps (20 mM), MgCl\(_2\), 6H\(_2\)O (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 \(\mu\)M and 10 \(\mu\)M in dimethyl sulphoxide), DiC\(_4\) (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\(_\alpha\)**

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 \(\mu\)g/ml), NAD (100 \(\mu\)M), \[^{32}\text{P}]\text{NAD} (30 \(\mu\)Ci/ml), NADP (1 mM), GTP (100 \(\mu\)M), saponin (100 \(\mu\)g/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\(_\alpha\) subunits were separated on SDS/polyacrylamide (11\%) gels and quantified as described above. The experiment shown is representative of ten others.

**Immunoprecipitation of G\(_\alpha\) from platelets**

Immunoprecipitation of G\(_\alpha\) 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 decapetide of rod transducin, and recognizes G\(_\alpha\) subunits, and the antiserum A569, which was produced in rabbits against the pentadecapeptide GAGES-GKSTIVKQMK that is located near the N-terminal region and recognizes all G\(_\alpha\) subunits.
with control and antiserum-incubated centrifugation

OMAT incubated was 45 min containing AS7 or Triton X-11amide saline. The added to Na2HPO4, A-Sepharose (1 1100 AM) radiography. four of blood pelleted were incubation of buffer both) were were continued in platelet-rich plasma (Fig. 1) and platelet-rich plasma [31].

RESULTS

Synergism of \( \alpha_2 \)-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 \( \mathbf{A}_2 \) 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, \( \mathbf{D} \mathbf{C}_8 \) induced a reversible aggregation response, whereas aggregation induced by PdBu was irreversible (Fig. 1). These effects closely correlate with the differential effects of \( \mathbf{D} \mathbf{C}_8 \) and PdBu on protein phosphorylation in platelets. Protein kinase C activation induced by \( \mathbf{D} \mathbf{C}_8 \) 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-\( \mathbf{O} \)-tetradecanoyl phorbol 13-acetate (TPA) was similarly effective, whereas the inactive parent compound 4\( \mathbf{z} \)-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 \( \alpha_2 \)-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 \( \alpha_2 \)-adrenoceptor agonist, and PdBu on platelets

Formation of \(^{32}\text{P} \) 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.

<table>
<thead>
<tr>
<th></th>
<th>Aggregation</th>
<th>ATP secretion</th>
<th>Phosphatidic acid (c.p.m.)</th>
<th>Protein phosphorylation (c.p.m. of (^{32}\text{P} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mm per deflection)</td>
<td>(( \mu \text{M} ))</td>
<td></td>
<td>47 kDa</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>2853 ± 328</td>
<td>745 ± 98</td>
</tr>
<tr>
<td>PdBu, 10 nM</td>
<td>16</td>
<td>0</td>
<td>2697</td>
<td>1346 ± 2</td>
</tr>
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<td>PdBu, 20 nM</td>
<td>26</td>
<td>2.5</td>
<td>2595</td>
<td>1888 ± 84</td>
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<tr>
<td>Adrenaline, 20 ( \mu \text{M} )</td>
<td>12</td>
<td>0</td>
<td>2995</td>
<td>763 ± 12</td>
</tr>
<tr>
<td>Adrenaline, 20 ( \mu \text{M} ) + PdBu, 10 nM</td>
<td>46</td>
<td>0</td>
<td>3108</td>
<td>1257 ± 45</td>
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<tr>
<td>Adrenaline, 20 ( \mu \text{M} ) + PdBu, 20 nM</td>
<td>112</td>
<td>4.0</td>
<td>2362</td>
<td>1908 ± 106</td>
</tr>
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<td>UK 14304, 20 ( \mu \text{M} )</td>
<td>4</td>
<td>0</td>
<td>2979</td>
<td>728 ± 25</td>
</tr>
<tr>
<td>UK 14304, 20 ( \mu \text{M} ) + PdBu, 10 nM</td>
<td>42</td>
<td>0</td>
<td>2734</td>
<td>1391 ± 51</td>
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<tr>
<td>UK 14304, 20 ( \mu \text{M} ) + PdBu, 20 nM</td>
<td>82</td>
<td>3.5</td>
<td>2403</td>
<td>1762 ± 18</td>
</tr>
</tbody>
</table>

\( \alpha_2 \)-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, \(^{32}\text{P} \)-labelled platelets (Fig. 2; similar results obtained with PdBu are now shown). The increase of phosphorylation has been quantified for the following \(^{32}\text{P} \)-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 \( \mu \text{M} \)) 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\(_{\alpha}\)**

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...

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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 \(^{32}\text{P} \)-phosphorylated platelet proteins. Experimental conditions were the same as described in legend to Fig. 1.
\[\alpha_2\]-Adrenoceptors and protein kinase C

Fig. 3. Time course of protein phosphorylation and phosphatidic acid formation in platelets exposed to adrenaline (10 \text{mM}) (\(\Box\)), PdBu (40 \text{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 \text{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.

This protein was present in platelet membranes and comigrated with the \(G_{\alpha}\) from bovine brain as well as the \[^{32}\text{P}\]-labelled, ADP-ribosylated \(G_{\alpha}\) from platelet membranes (results not shown). Since it has been reported that protein kinase C phosphorylates \(G_{\alpha}\) in the absence of \(\beta/\gamma\) subunits [40], we investigated whether the observed synergism of adrenaline and PdBu correlated with an increase of \(G_{\alpha}\) phosphorylation. We therefore performed immunoprecipitation studies using a \(G_{\alpha}\) antiserum (AS7) that specifically immunoprecipitated \[^{32}\text{P}\]-labelled, ADP-ribosylated \(G_{\alpha}\) from platelet membranes (Fig. 4). This platelet \(G_{\alpha}\) 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 \(G_{\alpha}\) (Fig. 4). Further experiments were carried out to immunoprecipitate \(G_{\alpha}\) after exposure of \[^{32}\text{P}\]-labelled intact platelets to adrenaline, PdBu or a combination of the two. It seemed that PdBu (50 \text{nM}) enhanced the \[^{32}\text{P}\]-labelling of \(G_{\alpha}\), but adrenaline did not increase further the protein kinase C-dependent phosphorylation of \(G_{\alpha}\) (results not shown). Since several phosphorylated proteins were found after gel separation of the immunoprecipitates, more rigorously controlled experiments were performed. Using the \(G_{\alpha}\)-antiserum AS7 or the \(G_{\alpha}\)-antiserum AS69, \(G_{\alpha}\) 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 \(G_{\alpha}\) but was not \(G_{\alpha}\). The phosphorylated protein was also present under conditions in which platelet \(G_{\alpha}\) was not immunoprecipitated (Fig. 5, and results not shown). It seems, therefore, that protein kinase C does not phosphorylate \(G_{\alpha}\) 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. 4. Immunoprecipitation of ADP-ribosylated \(G_{\alpha}\) from platelet membranes

Various amounts of \[^{[32}\text{P}][\text{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 \(G_{\alpha}\) was 15\% after immunoprecipitation.

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Fig. 5. Immunoprecipitation of G\(\alpha\) by G\(_{\alpha\text{sub}}\) antisem A569

Suspensions of washed platelets prelabelled with \(^{32}\text{P}\) 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 \(\mu\)l of saline (lanes a and d), 4 \(\mu\)l of affinity-purified G\(_\alpha\)-antisem A569 (lanes b and e) or 4 \(\mu\)l of affinity-purified G\(_\alpha\)-antisem that had been preincubated with 20 \(\mu\)g of the specific G\(_\alpha\)-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\(_\alpha\)/G\(_\alpha\) purified from bovine brain; lanes h and i, whole platelet suspensions (1 \(\mu\)l). The experiment is representative of two others.

G\(_\alpha\), we used guanabenz, which is a weak \(\alpha_2\)-adrenoceptor agonist for the aggregatory response but does not cause inhibition of adenylate cyclase [15,16]. Therefore, guanabenz does not interact with G\(_\alpha\) [11]. Guanabenz (10–100 \(\mu\)M) 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 \(\mu\)M) induced a small but significant increase of cytosolic Ca\(^{2+}\) from 86 ± 7 to 104 ± 6 nM (mean ± 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 \(\mu\)M) and adrenaline (20 \(\mu\)M) or UK 14304 (20 \(\mu\)M) that resulted in a maximal aggregation response did not cause an increase of \(^{32}\text{P}\)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 \(\mu\)M)-induced platelet shape change and aggregation of platelet-rich plasma did not inhibit aggregation induced by PdBu or PdBu plus adrenaline (results not shown).

### Table 2. Effects of adrenaline and PdBu on pertussis toxin-dependent ADP-ribosylation of G\(_i\)

<table>
<thead>
<tr>
<th>Time after addition of stimulus (s)</th>
<th>ADP-ribosylation (c.p.m. of (^{32}\text{P}))</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Adrenaline, 20 (\mu)M</td>
<td>1025 ± 20</td>
</tr>
<tr>
<td>PdBu, 50 nm</td>
<td>1045 ± 38</td>
</tr>
<tr>
<td>Adrenaline + PdBu</td>
<td>1060 ± 42</td>
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<tr>
<td>(\alpha)-Thrombin, 20 nm</td>
<td>1030 ± 32</td>
</tr>
</tbody>
</table>

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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 α₂-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, linked to α₂-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 α₂-adrenoceptor activation did not change the pattern and extent of protein phosphorylation induced by DiC₈ 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 α₂-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 α₂-adrenoceptor 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, α₂-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 $\alpha_2$-adrenergic receptor activation is the dissociation of $G_\alpha \times \beta/\gamma$ 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 $\alpha_2$-adrenergic receptors in platelets [45]. $\alpha_2$-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 $\alpha_2$-adrenergic agonist UK 4304 but no synergism with the partial $\alpha_2$-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].

$G_\alpha \times \beta/\gamma$ could possibly produce actions in platelets other than inhibition of adenylate cyclase. The possibility that $G_\alpha \times \beta/\gamma$ 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 $G_\alpha \times \beta/\gamma$ on SDS gels and was also present in immunoprecipitates obtained after incubating platelet lysates with antisem against $G_\alpha \times \beta/\gamma$. However, our results also show that this protein, which is phosphorylated by protein kinase C, is not identical to $G_\alpha \times \beta/\gamma$. 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 $\alpha_2$-adrenergic receptor 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, $\alpha_2$-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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REFERENCES


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