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The potentiation of phorbol ester-induced aggregation of human platelets by the prostaglandin endoperoxide analogue, U46619

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It was not possible to desensitize human blood platelets to 12-deoxyphorbol-phenylacetate (DOPP) stimulation in a manner analogous with that to platelet aggregating factor (PAF), prostaglandin-endoperoxide analogue (U46619) or adenosine diphosphate (ADP). Platelets previously desensitized to U46619, when challenged with DOPP and ADP, showed an increased aggregation and release of 5-HT. Sub-threshold aggregating doses of U46619 also caused a potentiation of the platelet response and release reaction to DOPP. The concentration of U46619 used to pretreat platelets affected the extent of potentiation of platelet stimulation induced by DOPP. The degree of potentiation was also affected by the time interval between addition of U46619 and DOPP. U46619 did not potentiate the aggregating effects of PAF, or ionophore A23187. The stimulus potentiation of DOPP by U46619 was abolished by prostacyclin (PGI_2) and an antibody to U46619, but was unaffected by indomethacin and CP/CPK.

The hydrolysis of phosphatidylinositol-1,4,5-trisphosphate by phospholipase C to produce the second messengers inositol-1,4,5-trisphosphate and diacylglycerol initiates a sequence of biochemical events leading to human platelet aggregation and secretion of 5-hydroxytryptamine (5-HT) from dense granules (Berridge 1984). This sequence may be described as a third pathway for the induction of platelet aggregation. Platelet agonists including thrombin, collagen, platelet aggregating factor (PAF) and phorbol ester (Hallam et al 1985; Sano et al 1983, 1985) are now known to induce phosphorylation of platelet proteins of molecular weight 20 K-Da and 40 K-Da, together with the mobilization of Ca^{2+} from internal stores. The 20 K-Da protein is associated with myosin light chains whilst the 40 K-Da protein has recently been identified as lipocortin an activator of phospholipase A_2 and the possible corticosteroid receptor site (Wallner et al 1986). Phorbol esters, the tumour-promoting constituents of plants of the families Euphorbiaceae and Thymelaeaceae, have been instrumental in the elucidation of this pathway (Aitken 1986). Tetradecanoylphorbol acetate (TPA) has been shown to substitute for diacylglycerol in the activation of the Ca^{2+}, phospholipid-dependent protein kinase C, resulting in phosphorylation of platelet proteins (Naga et al 1983).

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The induction of human platelet aggregation by phorbol esters was shown to be rigidly structurally dependent (Edwards et al 1983) and correlated with their tumour-promoting activity. 12-Deoxyphorbol-phenylacetate (DOPP) was the most potent of a number of phorbol derivatives tested for their ability to stimulate platelets (Westwick et al 1980). Recently these compounds have been shown to activate protein kinase C in a cell-free assay (Ellis et al 1985) and to induce phosphorylation of the 20 K-Da and 40 K-Da proteins of GH_3 cells in-vitro (Brooks et al 1986). However, the induction of platelet aggregation by phorbol esters differs from that effected by several other agonists, including PAF and thrombin, in that cytosolic Ca^{2+} mobilization was not a feature of TPA-induced aggregation (MacIntyre et al 1985a). Furthermore, thromboxane A_2 generation was minimal when platelets were stimulated to aggregate by DOPP (Williamson et al 1981). As part of our investigation into the mechanism of action of DOPP, we now describe the potentiation of DOPP stimulation of human platelets by the prostaglandin endoperoxide, U46619.

MATERIALS AND METHODS

Platelet preparation

Human venous blood from healthy volunteers who had received no medication in the previous 14 days was collected by venupuncture into 3·24% trisodium citrate (1 volume to 9 volumes of blood). Platelet rich

plasma (PRP) was obtained by centrifugation at 160*g* for 10 min at 22 °C. Platelet poor plasma (PPP) was obtained by further centrifugation at 27 000*g* for 20 min. The platelet content of the PRP was adjusted to 300 000 μL^{-1} with PPP. Aggregation was monitored with a Born Mk III aggregometer.

Platelet release reaction

Platelets were labelled with [^{14}C]5-HT by incubation of 10 mL of PRP with 1·5 μL of [^{14}C]5-HT (50 mCi $mmol^{-1}$) at 37 °C for 30 min. The release of [^{14}C]5-HT was determined by incubating stirred prelabelled platelets with the agonist for 4 min. 400 μL of PRP was then removed and added to 80 μL of cold 5% formaldehyde in EDTA (100 mM). This mixture was rapidly centrifuged in an Eppendorf centrifuge for 5 min. 100 μL of the contents were removed and added to 4 mL of scintillant in duplicate.

Desensitization to U46619

To stirred platelets, U46619 was added in an amount (200 ng mL^{-1}) insufficient to induce platelet aggregation. This amount was added several times (normally twice), and subsequent to this treatment, platelets would not respond to a larger dose of U46619.

Desensitization to DOPP

Attempts were made to desensitize platelets to DOPP as described above but platelets always responded to larger amounts of DOPP subsequently. Attempts were made at desensitization by maintaining DOPP (20 ng mL^{-1}) added to unstirred platelets at 37 °C for 20 min. Subsequent stirring of platelets with larger amounts of DOPP (400 ng mL^{-1}) still induced aggregation.

Desensitization to PAF

A dose of PAF (sufficient to induce maximal aggregation) was added to PRP without stirring. The mixture was left for 10 min and then used as previously described.

Materials

U46619 (15(*S*)-hydroxy-11α,9α-(epoxymethano)-prosta-5Z,13E-dienoic acid) and prostacyclin (PGI_2) were gifts from Dr J. E. Pike (Upjohn and Co., MI). U46619 at a concentration of 10 mg mL^{-1} in ethanol was stored at −20 °C. It was diluted with 0·9% NaCl (saline) as required. 12-Deoxyphorbol-13-phenylacetate (DOPP) was isolated from the fresh latex of *Euphorbia poissonii* as previously described (Schmidt & Evans 1979). DOPP was stored at a concentration of 1 mg mL^{-1} in acetone at −20 °C. Dilutions in acetone were made as required. Acetone 5 μL per 500 μL of PRP had no effect upon platelet aggregation.

Indomethacin was obtained from Merck, Sharp and Dohme (Herts UK); CP/CPK (creatine phosphate/creatine phosphokinase) was obtained from Sigma (Poole, UK); [^{14}C]5-hydroxytryptamine (5-HT) 50 mCi $mmol^{-1}$ was purchased from the Radiochemical Centre, Amersham, UK; platelet aggregating factor (PAF), Ionophore A23187 and antibody to U46619 were kindly provided by Dr J. Westwick.

RESULTS

DOPP-induced aggregation of human platelets

DOPP induced a dose related aggregation of human blood platelets that involved a significant release of 5-HT from dense granules (Fig. 1).

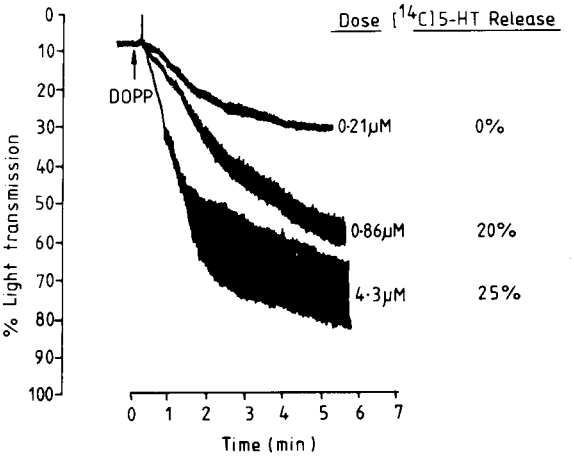

FIG. 1. Human blood platelet aggregation and 5-HT release induced by DOPP (0·21–4·3 μM).

Potentiation of DOPP-induced aggregation by U46619

Platelet-rich plasma (PRP) exposed to DOPP (10 ng mL^{-1}) for 20 min at 37 °C still responded to DOPP (0·21–4·3 μM) with aggregation, whereas it was possible to desensitize platelets to platelet aggregating factor (PAF, 1 μg mL^{-1}) and to prostaglandin endoperoxide (U46619, 200 ng mL^{-1}) by prior exposure of the platelets to PAF (500 ng mL^{-1}) and U46619 (200 ng mL^{-1}) (Fig. 2). Platelets previously desensitized to U46619 (200 ng mL^{-1}) still responded to DOPP (0·086–0·21 μM) with aggregation (Fig. 3) and release of 5-HT from dense granules. A similar effect was also observed when ADP (3 μM)

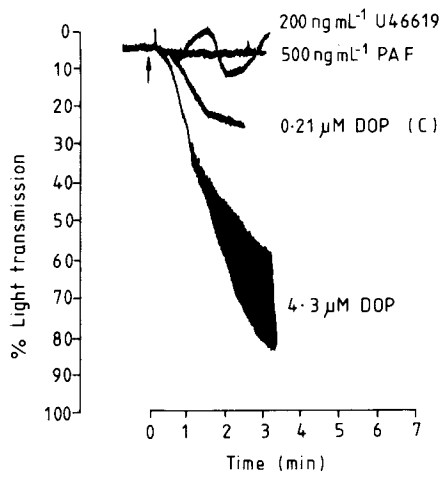

FIG. 2. (A) Attempted induction of aggregation by U46619 (200 ng mL^{-1}) on platelets previously exposed to U46619 (200 ng mL^{-1}). (B) Attempted induction of aggregation by PAF (500 ng mL^{-1}) on platelets previously exposed to PAF (1 μg mL^{-1}). (C) Aggregation of human platelets by DOPP (0·21 to 4·3 μM) previously exposed to DOPP (10 ng mL^{-1}).

was added to U46619 (200 ng mL^{-1})-desensitized platelets (Fig. 3).

The uses of subthreshold doses of U46619 (40 ng mL^{-1}) added to platelets followed by DOPP (0·086–0·21 μM) 2 min later also caused an increase in aggregation and 5-HT release (Fig. 4). The concentration of U46619 used to pretreat the platelets affected the degree of potentiation observed (Fig. 5).

Stability of U46619 in plasma

When U46619 (160 ng mL^{-1}) was incubated in plasma over 30 min, no significant difference in platelet response to U46619 was observed (Fig. 6). However, variation of the time over 1 to 10 min between the addition of U46619 and DOPP to platelets did affect the potentiation of platelet aggregation produced by DOPP (Fig. 7); this decreased with increase in time.

Effect of U46619 on other platelet agonists

Potentiation of platelet aggregation with DOPP on U46619 (200 ng mL^{-1})-pretreated platelets, as found with DOPP (0·86–0·21 μM) was also observed when adrenaline (0·6–1 μg mL^{-1}), or ADP 2–5 μM) were added to platelets pretreated with U46619 but not with the Ca^{2+} ionophore A23187 (20–40 μM) or PAF (10–20 ng mL^{-1}) (Fig. 8).

Antagonism of U46619-potentiation of DOPP

Potentiation of the effect of DOPP in U46619-pretreated platelets, as found with DOPP (0·86–0·21 μM) was antagonized by prostacyclin (PGI_2, 1–5 ng mL^{-1}) but not antagonized by a mixture of CP/CPK and indomethacin (CP/CPK,

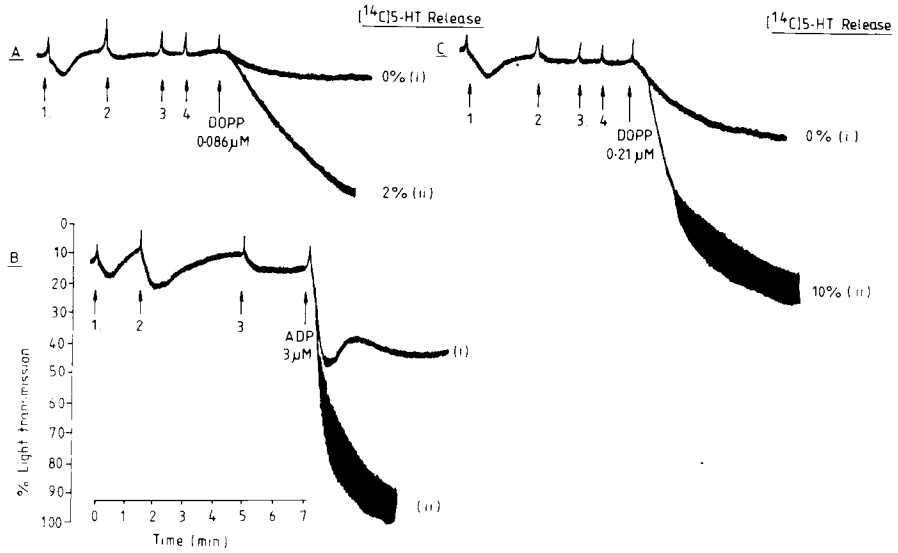

FIG. 3.(A) (i) The effect of DOPP (0·086 μM) on PRP aggregation and 5-HT release. (ii) The effect of DOPP (0·086 μM) on PRP aggregation and 5-HT release subsequent to exposure to 150, 150, 100 and 100 ng mL^{-1} of U46619. (B) (i) The effect of ADP (3 μM) on PRP aggregation. (ii) The effect of ADP (3 μM) on platelets exposed to U46619 200, 200 and 200 ng mL^{-1}. (C) (i) The effect of DOPP (0·21 μM) on PRP aggregation and 5-HT release. (ii) The effect of DOPP (0·21 μM) on PRP aggregation and 5-HT release subsequent to exposure to 150, 150, 100 and 100 ng mL^{-1} of U46619.

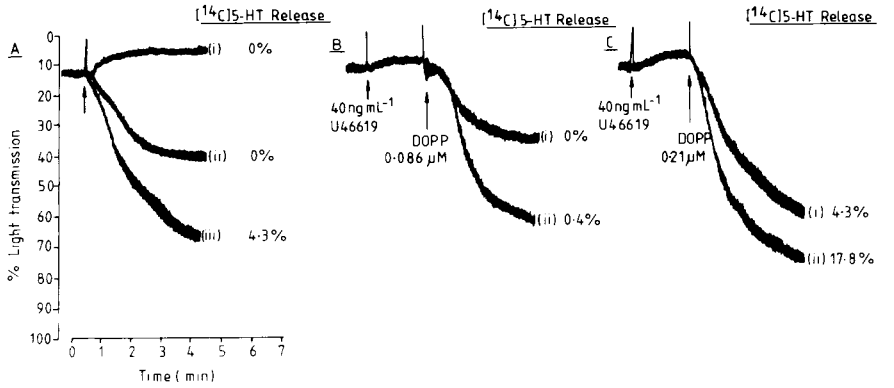

FIG. 4. (A) (i) The effect of U46619 40 ng mL^{-1}; (ii) DOPP 0·086 μM; (iii) DOPP 0·21 μM on human platelet aggregation and 5-HT release. (B) (i) The effect of 0·086 μM DOPP on human platelet aggregation and 5-HT release. (ii) The effect of 0·086 μM DOPP on platelet aggregation and 5-HT release 2 min after the addition of U46619 40 ng mL^{-1}. (C) (i) The effect of 0·21 μM DOPP on platelet aggregation and 5-HT release. (ii) The effect of 0·21 μM DOPP on platelet aggregation and 5-HT 2 min after the addition of U46619 40 ng mL^{-1}.

4 mM of 40 u mL^{-1} and indomethacin, 0·03 mM) (Fig. 9).

Potentiation of the effect of DOPP (0·21 μM) or ADP (2 μM) with U46619-pretreated platelets was abolished by 15 μL of an antibody to U46619 (14 ng mL^{-1} in saline) (Fig. 10). The effect, therefore, may be identified as membrane centred and U46619 must be present in plasma.

DISCUSSION

12-Deoxyphorbol-phenylacetate (DOPP) induced a dose-related aggregation of human blood platelets, involving a significant release of 5-HT from dense granules (Fig. 1). DOPP, together with other phorbol esters, has been shown to activate protein kinase C in a cell-free assay (Ellis et al 1985) and to induce phosphorylation of the 20 K-Da and 40 K-Da proteins of GH_3 cells in-vitro (Brooks et al 1986). The related C12,13 diester, tetradecanoylphorbol acetate (TPA), also induces platelet aggregation (Zucker et al 1974) and activation of protein kinase C resulting in the phosphorylation of myosin light chains (Naga et al 1983). The induction of human platelet aggregation and a release of 5-HT via this pathway is not dependent upon a C-12 acyl group in the tigliane nucleus. However, the presence of an ester moiety at either C-12 or C-13 is necessary for platelet stimulation in that our present results have shown the parent alcohol, phorbol, to be inactive. Human platelets could be desensitized to stimulation

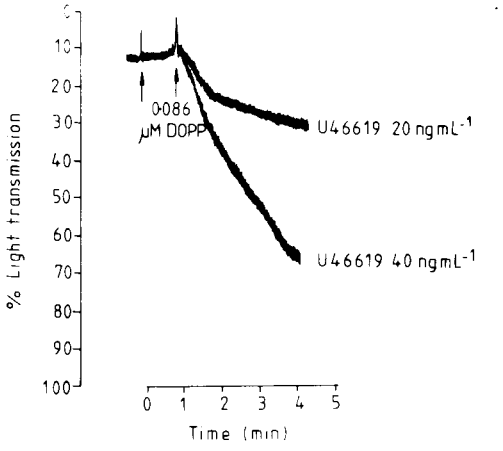

FIG. 5. The effect of U46619 (20–40 ng mL^{-1}) administered 1 min before the DOPP (0·086 μM) on human platelet aggregation.

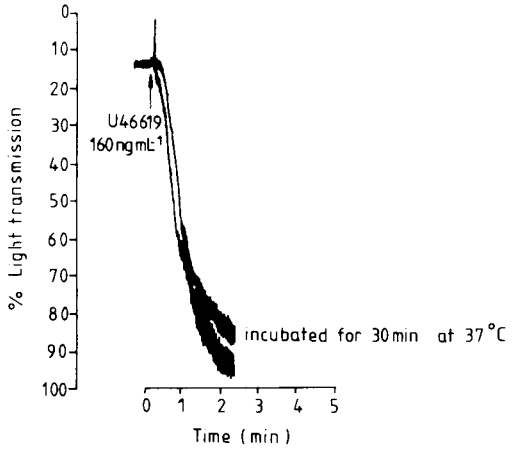

FIG. 6. The effect of incubation of U46619 (160 ng mL^{-1}) in plasma at 37 °C on the aggregation by U46619 of human platelets.

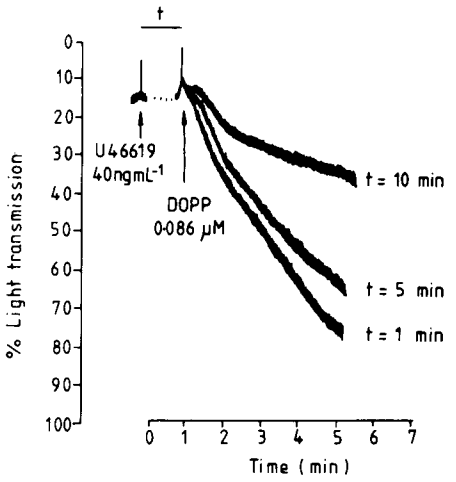

FIG. 7. The effect of variation of time (t = min) between the addition of U46619 (40 ng mL^{-1}) and DOPP 0·086 μM on the potentiation of DOPP-induced platelet aggregation.

by platelet aggregating factor (PAF) and to the endoperoxide analogue U46619 by previous exposure to 1 μg mL^{-1} and 200 ng mL^{-1} of PAF and U46619, respectively (Fig. 2a, b). Prior exposure of platelets to DOPP (10 ng mL^{-1}) did not result in a desensitization of those platelets to further stimulation by DOPP (0·21 μM, Fig. 2c), suggesting that DOPP-induced aggregation may be more complex than previously realized. U46619 is believed to induce platelet aggregation by interaction at a thromboxane A_2/endoperoxide membrane receptor leading to the suppression of adenylate cyclase and a reduction in platelet levels of cyclic AMP (cAMP), together with mobilization of intracellular Ca^{2+} ions (Hatmi et al 1986). PAF induces increased production of the platelet second messengers, inositol trisphosphate and diacylglycerol, and subsequent protein kinase C activation and Ca^{2+} flux (MacIntyre et al 1985b). Our results demonstrate that the platelet receptor sites to U46619 and PAF can become refractory to their respective agonists in a manner similar to that previously demonstrated with 5-HT, ADP and vasopressin (Ruggles & Scrutton 1979; Evans & Gordon 1974).

The platelet phorbol ester receptor does not undergo a refractory period on exposure to DOPP. The essential structural features for phorbol ester stimulation of platelets include a C-20 primary hydroxyl group as well as an acyl group at C12/13 and these features confer an amphiphatic nature on phorbol derivatives (Westwick et al 1980). Furthermore, DOPP is a rigid molecule, stable in plasma at 37 °C (Williamson et al 1981), and it is likely that it would associate with membrane phospholipids. DOPP would therefore perturb the phospholipid substrate in the vicinity of protein kinase C leading to a loss of a refractory period for that receptor.

Platelets previously desensitized to U46619 still responded to DOPP at 0·086–0·21 μM (Fig. 3a, c) with an increased response both in terms of aggregation and release of 5-HT from dense granules. ADP (3 μM) in the same circumstances behaved similarly (Fig. 3b). Pretreatment of platelets with subthreshold doses of U46619 (40 ng mL^{-1}) also induced an increase in aggregation and 5-HT release when DOPP (0·086—0·21 μM, Fig. 4b, c) was used as a challenging agent. The enhancement of DOPP-induced aggregation by U46619 was dose-dependent in that platelets pretreated with 40 ng mL^{-1} of U46619 exhibited a greater increase in potentiation of 0·086 M DOPP-induced aggregation compared with pretreatment with 20 ng mL^{-1} U46619 (Fig. 5). The lowering of platelet cAMP levels by exposure to U46619 led to an enhancement of the effects of platelet protein kinase C activated by DOPP.

U46619 was shown to be stable in plasma by the fact that after incubation at 37 °C for 30 min there was no significant difference in platelet response to it (Fig. 6). Nevertheless, its effects on platelet cAMP levels decreased with time. Significant differences were observed with time between 1–10 min in its potentiation of DOPP (0·086 μM)-induced platelet aggregation (Fig. 7).

Adrenaline and ADP are both platelet agonists which lower cAMP levels during aggregation (Salzmann 1974; Jakobs 1978). The synergistic effects on platelet aggregation and 5-HT release shown by U46619 and DOPP (Fig. 8a) were also seen when adrenaline (0·6–1 μg mL^{-1}) and ADP (2 to 5 μM) (Fig. 8b, c) were added to platelets previously exposed to 100 ng mL^{-1} of U46619. Protein kinase C activation by the minimal dose of DOPP may therefore mimic the effects of adrenaline and ADP upon the adenylate cyclase/cAMP system of platelets. This effect was not observed when ionophore A23187 or PAF were used as agonists on U46619-pretreated platelets (Fig. 8d, e). Diacylglycerol, generated by PAF stimulation of phospholipase C, is probably bioregulated, maybe by means of other *sn*-1,2-diacylglycerols (Lapetina et al 1985). Despite the fact that Ca^{2+} mobilization from internal stores is not a feature of phorbol ester stimulation of platelets (MacIntyre et al 1985a), DOPP activates protein kinase C, even at minimal doses, and will act synergistically with U46619 on human platelet aggregation.

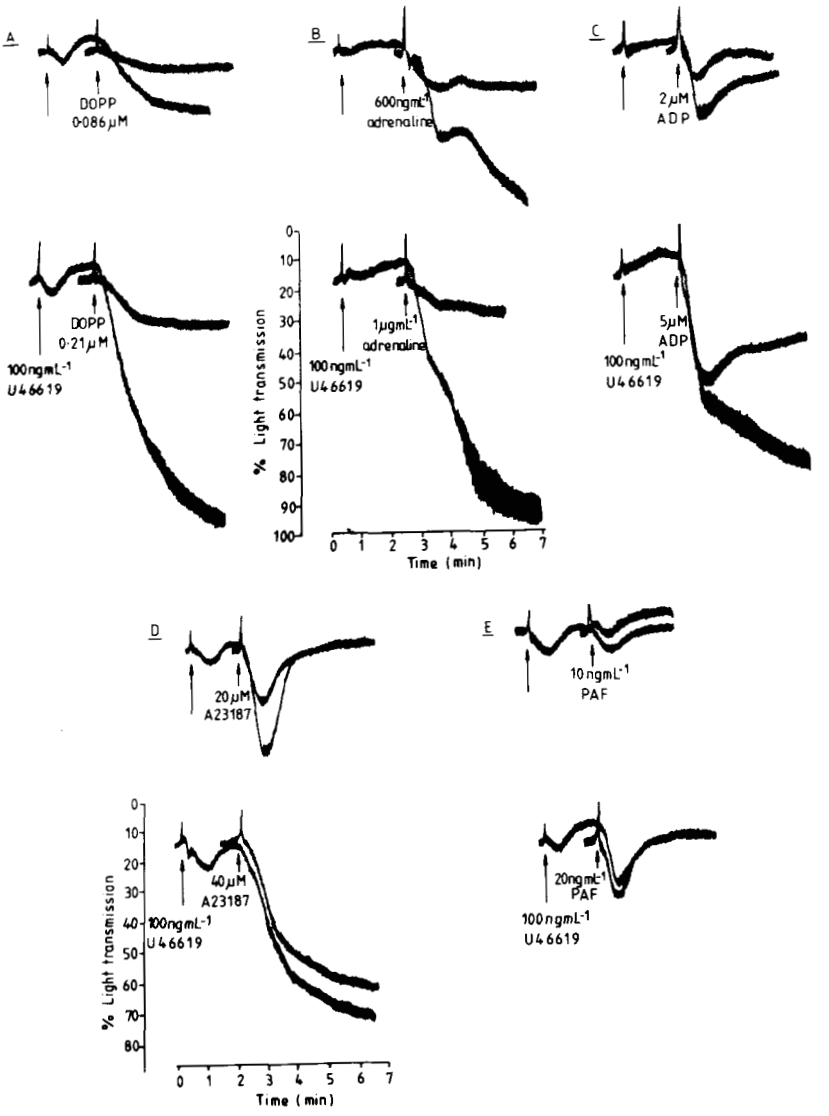

FIG. 8. The effect of pretreatment of platelets with U46619 (100 ng mL^{-1}) at the first arrow on the aggregation induced by (A) DOPP (0·086–0·21 μM), (B) adrenaline (0·6–1 μg mL^{-1}), (C) ADP (2–5 μM), (D) A23187 (20–40 μM), (E) PAF (10–100 ng mL^{-1}).

It has been shown (Westwick et al 1980; Williamson et al 1981) that DOPP stimulation of platelets involves only a small release of thromboxane B_2 and that phospholipid turnover is not a feature of the DOPP-induced aggregation (Evans & Edwards 1986). The potentiation of DOPP-induced aggregation by subthreshold doses of U46619 was unaffected by indomethacin (0·03 mM) (Fig. 9b). The synergistic effect was also unaffected by CP/CPK, an ADP scavenger system, suggesting that the potentiation of DOPP by U46619 was not mediated by either arachidonate or ADP release from platelets. The potentiation was abolished by prostacyclin (PGI_2, 50 ng mL^{-1}, Fig. 9a) further supporting the concept that the synergistic actions of DOPP and U46619 were involved with critical levels of platelet cAMP. The effect of U46619 on DOPP-induced platelet aggregation is considered to be mediated by means of a membrane receptor, U46619, being present in platelet plasma, since an antibody inhibited the

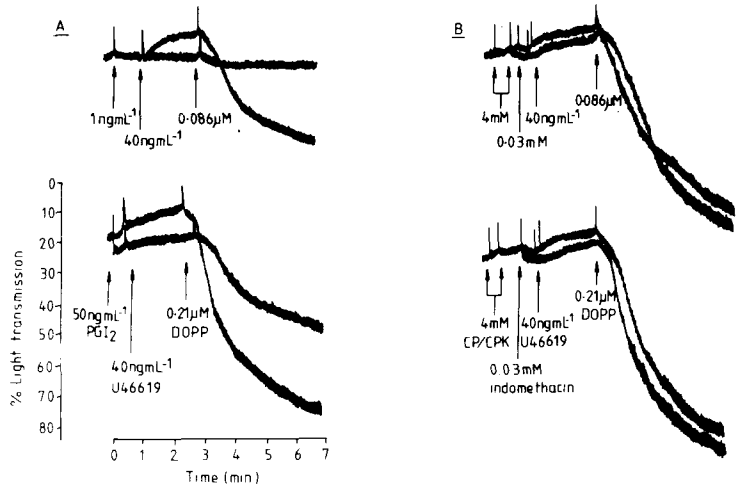

FIG. 9. (A) The effect of PGI_2 (1 to 50 ng mL^{-1}) on the U46619 (40 ng mL^{-1}) potentiation of aggregation induced by DOPP (0·086–0·21 μM), (B) The effect of CP/CPK (4 mM) and indomethacin (0·03 mM) on the U46619 (40 ng mL^{-1}) potentiation of aggregation induced by DOPP (0·086–0·21 μM).

potentiation when added to PRP (15 μL of a 14 ng mL^{-1}) solution (Fig. 10a). This was also noted when U46619 potentiated ADP-induced aggregation (Fig. 10b).

It has recently been demonstrated that the phorbol ester, TPA, has no effect upon the catalytic moiety of adenylate cyclase or on the stimulatory N_S-protein and the PGE_1 receptor (Jakobs et al 1985). However,

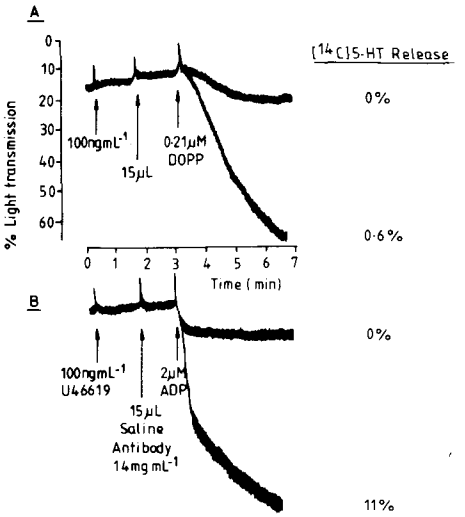

FIG. 10. (A) The effect of antibody to U46619 (15 μL of a 14 ng mL^{-1} solution), administered at the second arrow, on U46619 (100 ng mL^{-1}), administered at the first arrow, potentiation of aggregation induced by DOPP (0·21 μM) and 5-HT release. (B) The effect of antibody to U46619 (15 μL of a 14 mg mL^{-1} solution), administered at the second arrow, on U44619 (100ng mL^{-1}), administered at the first arrow, potentiation of aggregation induced by ADP (2 μM) and 5-HT release.

phorbol ester stimulation of protein kinase C may interfere with the adenylate cyclase system of platelets by alteration of N_i-protein-mediated signal transduction, thereby explaining the enhancement of platelet aggregation when low doses of U46619 and DOPP are added consecutively to platelet-rich plasma.

Acknowledgements

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