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Secretion and properties of a polypeptide factor generated by phorbol ester stimulation of human blood platelets

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12-Deoxyphorbolphenylacetate (DOPP) was shown to be the most potent of a range of hydroxy- and deoxy-phorbol esters in terms of induction of human platelet aggregation [1, 2]. This compound is also a known tumour promoting agent [3] *in vivo* and will activate the phospholipid and calcium-dependent protein kinase C [4] in a cell free assay. Protein kinase C is believed to be the phorbol ester receptor site [5] and one member of this series of compounds, tetradecanoylphorbolacetate (TPA), has been shown to induce platelet aggregation which may be associated with myosin light chain phosphorylation [6].

The direct aggregating effect of DOPP on platelets is followed by a small release reaction from dense granules together with the release of a transferable aggregating substance (TAS) [7, 8]. As part of our investigations into phorbol ester induction of platelet aggregation we have investigated the production and properties of TAS following platelet aggregation induced by DOPP.

Materials and methods

Reagents. Pinane thromboxane A_2 was provided by Dr J. B. Smith (Philadelphia, PA), prostacyclin (PGI_2) by Dr J. E. Pike (Upjohn Co., MI) and β-γ-methylene ADP, platelet aggregating factor (PAF) and phenidone by Dr J. Westwick (London, U.K.). Verapamil was purchased from Roche (Basle, Switzerland), EDTA and trypsin from Sigma (U.K.), *p*-bromophenacylbromide from BDH (Poole, U.K.), trifluoperazine from Smith, Kline & French (U.K.), indomethacin from Merck, Sharp and Dohme (Rathway, U.S.A.) and promethazine from May and Baker (Dagenham, U.K.).

12-Deoxyphorbolphenylacetate (DOPP) was isolated from the fresh latex of *Euphorbia poissonii* [9]: human venous blood was collected from healthy male donors, platelet-poor plasma (PPP) and platelet-rich plasma (PRP) were prepared as previously described [8]. Rabbit blood was collected from the marginal ear vein of New Zealand White laboratory animals and PPP and PRP prepared as above.

Secretion of TAS. A Born Mk. III aggregometer was used to monitor the platelet aggregations. Acetone was used as the solvent for DOPP and this solvent had no effect upon platelets at a maximum concentration of 0.5% in PRP. Aggregation was quantified by determining the maximum aggregation obtained within 4 min. A dose–response curve was obtained for DOPP induced aggregation and the minimum dose of DOPP that induced aggregation was found to be 0.09 μM. DOPP (0.43 or 0.86 μM) was added to 500 μl of PRP in an aggregometer cuvette and 100 μl of PRP was removed at intervals from 30 sec. to 1 hr and added to 400 μl of fresh recipient PRP. The degree of aggregation of the recipient PRP was determined as before.

Inhibition of TAS production. Phenidone (0.5 mM in ethanol) or trypsin (20 μl of 0.1 mg/ml in phosphate buffer saline at pH 7.4) were added to 500 μl of PRP 1 min before the addition of 0.86 μm DOPP. After 4 min 100 μl of PRP was transferred to recipient PRP and aggregation monitored as above.

Inhibition of TAS induced aggregation. TAS was produced by the addition of either 0.43 or 0.86 μM of DOPP to PRP. To the recipient platelets inhibitors dissolved in ethanol or acetone were added 1 min before the introduction of 100 μl of donor PRP. The concentrations of inhibitors used is given in Table 1.

Response of stored platelets. PRP was stored for 24 hr at −4°, and DOPP-induced aggregation carried out as before.

Storage of TAS. 500 μl of PRP subsequent to DOPP-induced aggregation (0.86 μM) was centrifuged at 2700 *g* for 30 min and the supernatant plasma was stored at −4° for 24 hr. Quantities of plasma (100 μl) were used to induce aggregation in 400 μl of recipient platelets as before.

Washed platelet system. Whole human blood (50 ml) was mixed with 7.5 ml of ACD (1.4% citric acid, 2.5% trisodium citrate and 2% glucose). The pH was adjusted to 6.5 with sodium hydroxide, centrifuged at 650 *g* for 10 min and the pH of the PRP adjusted to 6.5 with 110 mM citric acid. EDTA (100 μl, 1 mM per 10 ml) was added. The suspension was recentrifuged at 2500 *g* for 10 min and the platelets suspended in citric acid buffer pH 6.5; EDTA (1 mM) and PGE_1 (7 ng/ml) were added. The suspension was recentrifuged for 7.5 min and the pellet resuspended as before. This suspension was again centrifuged and resuspended in a solution of KCl (0.0373%), NaCl (0.671%), $CaCl_2$ (0.0294%), $MgCl_2$ (0.0203%) and Tris (0.375%). To this was added 90 mg/100 ml of glucose and the pH adjusted to 7.4. The suspended washed platelets were used for aggregation studies (500 μl) as previously described for PRP. These aggregations were repeated upon the addition of 10 μl of 1.01% $MgCl_2$, 100 mM $CaCl_2$ and 50 mg/ml of fibrinogen to the agregometer cuvette.

Desensitisation of platelets to PAF. PRP was produced as before and without stirring PAF was added sufficient to induce a maximal aggregation. After standing for 10 min the PRP was used as above.

Results and discussion

The tumour promoting phorbol ester [3] DOPP, induced human platelet aggregation with an ED_{50} of 0.6 μM. We have previously demonstrated that this aggregation involves only a small release reaction from dense granules together with the secretion of a biologically active substance into plasma [8]. The first indication that phorbol ester stimulation would result in secretion of biologically active material into plasma subsequent to platelet aggregation was provided by Mufson and others [10], in that transferred plasma caused contraction of smooth muscle. We have confirmed that secretion of transferable aggregating substance (TAS) is dependent upon platelet aggregation and additionally that this secretion was not noted when PAF, thrombin or collagen were used as agonists.

The production of TAS was time-dependant and reached its maximum within 4 min of platelet aggregation induced by DOPP (Fig. 1). The half-life of TAS was found to be 20 min in plasma at 37°. Centrifugation of aggregated PRP indicated that TAS was secreted into plasma and was not platelet-bound, it was nevertheless, platelet-derived because washed platelets stimulated with DOPP in the normal manner also secreted TAS activity into the buffer solution (Fig. 2). The aggregation of human platelets by TAS was a true aggregation and not an agglutination, EDTA and PGI_2 both inhibited TAS induced aggregation (Table 1). Furthermore, the secretion of TAS was also found to be species-dependent in that, although DOPP was shown to be a potent stimulator of rabbit platelets, attempts to demonstrate transfer aggregation of recipient rabbit platelets failed.

Previous work [8] has shown that DOPP is not metabolised in plasma, and it is unlikely that the activity of TAS containing plasma was due to a phorbol ester metabolite. Furthermore, the transfer of a subthreshold dose of DOPP (0.09 μM) would be insufficient to account for TAS-induced aggregation. TAS does have properties in common with DOPP. Compounds (Table 1), which have been shown to inhibit DOPP-induced aggregation [8], such as calcium/calmodulin and phospholipase A_2 antagonists will also inhibit TAS-induced aggregation.

Accordingly, the receptor to TAS may be associated with the platelet membrane, as is the case for phorbol esters. We have also demonstrated that verapamil, an intracellular calcium antagonist (Table 1), had no effect upon either DOPP- or TAS-induced platelet aggregation at concentrations previously shown to prevent cytosolic calcium mobilisation [11]. There were, however, marked differences between DOPP- and TAS-induced aggregation of human blood platelets when compared in the washed platelet system. In this system DOPP only produced a full aggregation when calcium and magnesium ions together with fibrinogen were added, whereas this was not the case for TAS-induced aggregation (Fig. 2). A further distinction between the two agonists was the observation that PRP stored at 37° for 4 hr failed to respond to TAS whilst still fully responding to DOPP.

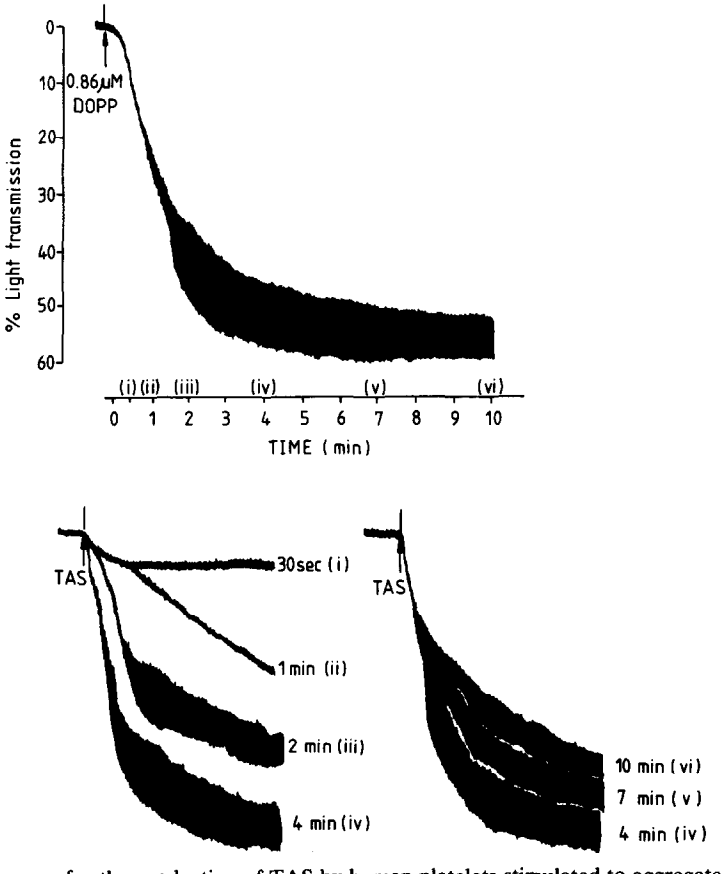

Fig. 1. Time course for the production of TAS by human platelets stimulated to aggregate by 0.86 μM DOPP; 100 μl of plasma removed at time intervals (i) to (iv) and added to 400 μl quantities of recipient PRP.

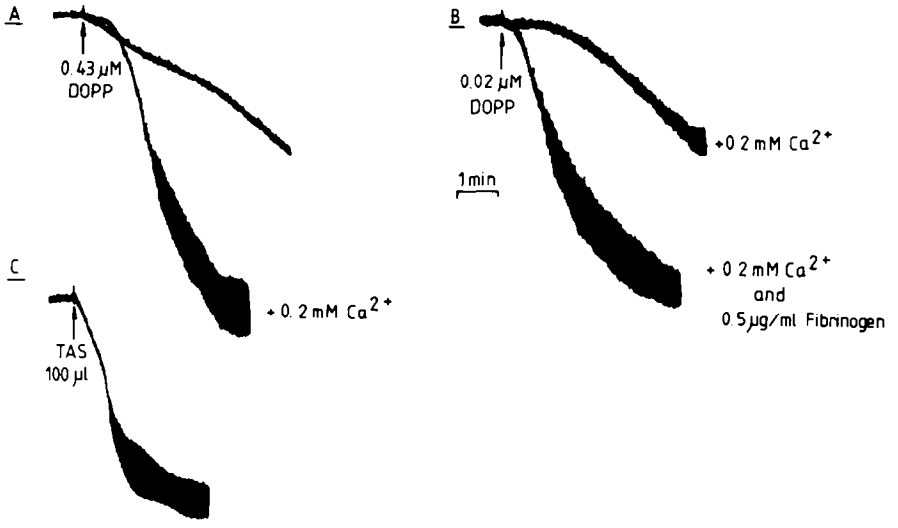

Fig. 2. A. The action of DOPP (0.43 μM) on washed platelets. B. The action of DOPP (0.02 μM) on washed platelets with and without the addition of fibrinogen 0.5 μg/ml). C. The action of TAS (100 μl) on washed platelets in the presence of 0.2 M calcium ions.

Table 1. A. Compounds which abolished the ability of TAS (100 μl) produced by platelet stimulation by DOPP (0.86 μM) to induce aggregation of recipient PRP. B. Compounds which failed to abolish the aggregating activity of TAS produced as in A

Compound	Concentration
A.	
EDTA	1.0–5.0 mM
PGI_2	1.0–5.0 ng/ml
p-Bromophenacylbromide	0.01–0.20 mM
Trifluoperazine	0.04–0.20 mM
Propranolol	1.44 μM
Imipramine	1.44 μM
promethazine	0.006–0.07 mM
indomethacin	0.03–0.1 mM
Trypsin	20 μl of 0.1 mg/1 ml solution
B.	
Pinane thromboxane A_2	0.001–0.04 mM
Verapamil	0.003–0.3 mM
β-γ-methylene ADP	0.02–0.1 mM

The cyclo-oxygenase inhibitor, indomethacin, did not antagonise DOPP-induced aggregation and neither did it affect DOPP stimulation of TAS secretion. Phenidone at concentrations previously reported to be both a cyclo-oxygenase and a lipoxygenase inhibitor also had no effect upon the secretion of TAS by platelets, and it is unlikely that TAS production was associated with arachidonate metabolism in platelets. These results were confirmed by the observation (Table 1), that the endoperoxide/thromboxane receptor antagonist, pinane thromboxane A_2 at a concentration of up to 0.004 mM did not antagonise TAS stimulation of platelets. It was also unlikely that TAS activity was associated with platelet generation of ADP in that β-γ-methylene-ADP an ADP receptor antagonist had no effect upon TAS-induced platelet aggregation (Table 1). The possibility remained that TAS was phospholipid derived, maybe resulting fromn phospholipase C stimulation. Phorbol esters have been shown to activate this enzyme in human neutrophils [12]. In this study we have desensitised human platelets to the ether linked phospholipid PAF. Platelets desensitised to PAF still responded to DOPP and TAS was secreted into plasma. Furthermore PAF-densensitised platelets also responded to stimulation by TAS (Fig. 3), and on that basis it was unlikely that TAS was related to that class of compound.

Attempts to purify TAS from plasma using conventional thin-layer and column methods of chromatography with silica gel as the adsorbant were unsuccessful. TAS activity was also lost when plasma containing this activity was stored at −4° for 24 hr suggesting that TAS might be a polypeptide. To investigate this suggestion further PRP was incubated with trypsin for 1 min prior to the addition of 0.43 μM DOPP, and 4 min later 100 μl of plasma were transfered to 400 μl of recipient platelets. The response of TAS was greatly reduced (Fig. 4).

The aggregation of human platelets induced by DOPP, a tumour promoting phorbol ester was found to be accompanied by the secretion of TAS. TAS appeared in

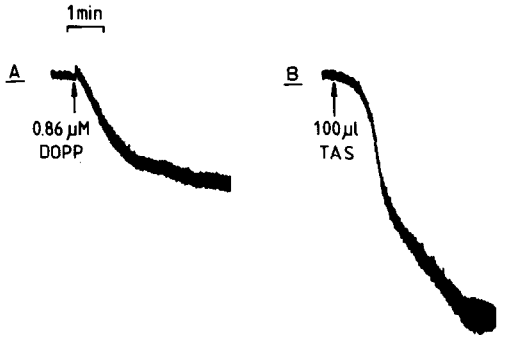

Fig. 3. A. Aggregation of human platelets previously desensitised to PAF by 0.86 μM DOPP. B. Aggregation of platelets previously desensitised to PAF by 100 μl of TAS.

plasma within 30 sec of aggregation and its production was maximal within 4 min with a half-life of 20 min. Washed human platelets which failed to respond fully to DOPP stimulation in terms of aggregation still secreted TAS into buffer solution. The aggregating activity of TAS was shown to be distinct in its properties from that of DOPP, ADP, PAF and the products of arachidonate metabolism. TAS could not be purified by conventional chromatography on silica gel as adsorbant and lost its activity as a result of freezing for 24 hr and by prior incubation with trypsin, accordingly it is probable that TAS is a polypeptide.

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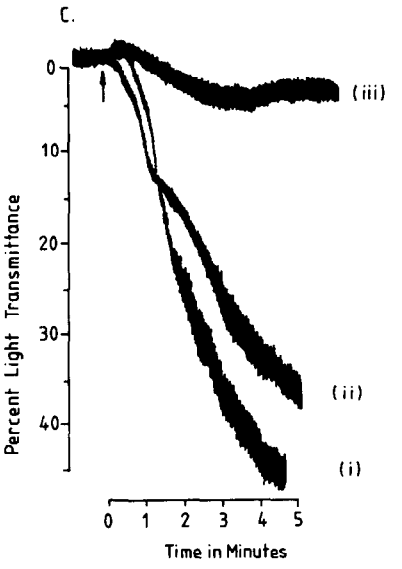

Fig. 4. (i) Aggregation of human platelets induced by 0.43 μM DOPP added to PRP 1 min following the addition of trypsin (0.1 mg/ml). (ii) Aggregation induced by TAS (100 μl) following secretion induced by DOPP (0.43 μM). (iii) The effect of 100 μl of plasma from (i) on recipient PRP.

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Metabolic activation of the tricyclic antidepressant amineptine by human liver cytochrome P-450

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Amineptine is widely used in several countries. Like other tricyclic antidepressants [1, 2], amineptine may produce hepatitis in some patients [3, 4]. We recently reported that amineptine is transformed by hamster liver cytochrome P-450 into a chemically reactive metabolite that may covalently bind to hepatic proteins both *in vitro* and *in vivo* [5, 6].

Cytochrome P-450 isozymes present in human liver are not exactly the same as those present in animals [7]. It was therefore of interest to see whether a similar metabolic activation also occurs with human liver microsomes.

Materials and methods

Chemicals. Amineptine hydrochloride and [11-^{14}C]-amineptine hydrochloride were generously given by Servier laboratories (Neuilly, France). The radiochemical (24 mCi·mmol^{-1}), labelled on the middle ring of the tricyclic structure, was prepared by Commissariat à l'Energie