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# STUDIES ON THE MECHANISM OF ACTION OF 12-DEOXYPHORBOLPHENYLACETATE, A POTENT PLATELET AGGREGATING TIGLIANE ESTER

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**Abstract**—12-Deoxyphorbolphenylacetate (12-DOPP) induced human platelet aggregation which was dependent upon the presence of divalent cations, the intracellular level of cyclic-AMP and an intact microtubular system, in common with other aggregating agents. However platelet secretion and thromboxane (Tx)$B_2$ synthesis did not contribute to 12-DOPP induced platelet aggregation as neither the Tx/endoperoxide antagonists pinane $TxA_2$ and trimethoquinone, the thromboxane synthetase inhibitors clotrimazole and 9,11,aza-prosta-5-13-dienoic acid nor the cyclo-oxygenase inhibitor indomethacin inhibited 12-DOPP induced aggregation. Furthermore the free radical scavengers aminopyrine, thioanisole and butylated hydroxy-toluene, the lipoxygenase inhibitor phenidone and the leucotrienes B and C antagonist FPL 55712 failed to modify 12-DOPP-induced aggregation.

Compounds which are thought to act as phospholipase inhibitors (bromophenacylbromide, mepacrine and propranolol as well as imipramine, desmethylimipramine, promethazine and trifluoperazine) which have been shown to selectively bind calmodulin, were found to be effective inhibitors of 12-DOPP induced aggregation. Aggregation induced by 12-DOPP involves a direct effect upon platelets followed by the release of unknown substances, probably phospholipid products. The released substances were shown to induce further aggregation of platelets. The aggregation induced by 12-DOPP is thus distinct from that induced by ADP, collagen, adrenaline and prostaglandin endoperoxides.

12-Deoxyphorbolphenylacetate (12-DOPP) is one of the most potent pro-inflammatory agents to have been isolated from the latex of plants from the genus *Euphorbia* [1]. This compound belongs to a relatively new but nevertheless large group of natural toxins known chemically as the tigliane esters [2]. Also included in this class of compounds is the well-known tumour-promoting agent tetradecanoylphorbol-acetate (TPA), originally isolated from the seed oil of *Croton tiglium* [3].

It has been shown [4] that submicrogram doses of TPA cause two stage aggregation of human blood platelets. Reports of the effects of TPA on platelet ultrastructure [5, 6] have shown that in contrast to ADP, TPA acted on channels of the open canalicular system and storage granules, rather than on the platelet discoid shape. We have previously confirmed earlier reports concerning the platelet aggregating ability of TPA [7] and further extended this study to observations of platelet aggregating ability of ten closely related pro-inflammatory tigliane and daphnane esters [8]. Structural features necessary for activity on platelets included an ester function at C-13 of the nucleus and a free primary hydroxy group at C-20. An ester moiety at C-12 as with TPA was not essential for potent aggregating ability. Of the series of 12-deoxyphorbol esters 12-DOPP was the most potent blood platelet aggregating agent, inducing aggregation at a concentration of 0.10 $\mu$m. Such structure activity studies are of significance because of the marked differences in pro-inflammatory activities of these esters [9]. Furthermore TPA is both a pro-inflammatory and tumour-promoting agent whilst 12-DOP esters have little if any tumour-promoting activity [10]. From *in vitro* comparisons of tumour-promoting and non-promoting esters it has been suggested that tigliane esters may interact at different receptor sites [11]. The study of the mechanism of action of the non-promoting but pro-inflammatory agent 12-DOPP on platelet aggregation may therefore be of greater significance than a study of the effects of TPA, because of the broader spectrum of activity of the latter substance.

Although from earlier studies [8] it is likely that a tigliane receptor exists on platelets, it has yet to be determined whether the esters induce release of dense granules or whether they activate arachidonic acid metabolism. TPA has been shown to mobilise phospholipid metabolism in other cells [12, 13]. This paper describes an investigation of the release of 5-HT and generation of thromboxane $B_2$ ($TxB_2$) after 12-DOPP stimulation, together with a study of the mechanism of 12-DOPP induced aggregation by the use of pharmacological antagonists and enzyme inhibitors.

## MATERIALS AND METHODS

*Blood collection.* Human venous blood was collected from healthy male donors, who had denied any medication in the previous 14 days. Nine volumes of blood were mixed with 1 vol of 3.24 per cent trisodium citrate.

*Platelet rich plasma (PRP) and platelet poor plasma (PPP).* Citrated blood was centrifuged at 160 *g* for 10 min at 22° to obtain PRP. PPP was obtained by further centrifugation at 2700 *g* for 20 min at 4°. The platelet content of the PRP was adjusted to 300,000

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per μl with PPP. The PRP tube was capped and stored at 37° for the duration of the experiment and was not used within 30 min of preparation [14].

*Reagents.* Prostaglandin (PG)$E_1$, $PGI_2$, 9-11-aza-prosta-5-13-dienoic acid were gifts from Dr. J. E. Pike (Upjohn Co., MI). Promethazine hydrochloride was a gift from May and Baker (Dagenham, U.K.) and trifluoperazine was a gift from Smith, Kline and French (Welwyn Garden City, U.K.). Pinane thromboxane $A_2$ ($PTA_2$) and carbocyclic thromboxane $A_2$($CTA_2$) were kindly provided by Dr. J. B. Smith (Philadelphia, PA). Aminopyrine and thioanisole were purchased from Aldrich Chemicals (Gillingham, U.K.); butylated-hydroxytoluene from BDH (Enfield, U.K.); imipramine, desmethylimipramine from Geigy (Cheshire, U.K.); indomethacin from Merck, Sharp and Dohme (Herts, U.K.); propranolol from I.C.I. (Cheshire, U.K.); ADP, EDTA, yohimbine, imidazole and colchicine from Sigma (Poole, U.K.); verapamil and trimethoquinone from Roche Products (Basle, Switzerland). $^{14}$C-5-OH-tryptamine (5HT) 50 mCi/mmole was obtained from the Radiochemical Centre (Amersham, U.K.); $^3$H-$TxB_2$ 120 mCi/mmole was obtained from New England Nuclear Laboratories. 12-Deoxyphorbolphenyl acetate (12-DOPP) was isolated from the fresh latex of *Euphorbia poissonii* Pax and its structure elucidated as previously described [15].

*Platelet aggregation.* A Born Mk. III aggregometer was used which was set at 0 per cent light transmission with 600 μl of PRP and 100 per cent light transmission with 600 μl PPP. The effect of each compound on 12-DOPP induced aggregation was studied by the addition of compound or vehicle 1 min before the addition of 12-DOPP. Aggregation was quantitated by determining the maximum percentage light transmission obtained within 4 min.

*Platelet release reaction.* Platelets were labelled with $^{14}$C-5HT as described by Mills and Roberts [16]. The release of $^{14}$C-5HT was determined by incubating stirred prelabelled platelets in an aggregometer cuvette with 12-DOPP for 4 min, then 500 μl of PRP was removed and added to 50 μl of 100 mM ETDA and 175 μM indomethacin in Eppendorf tubes in an ice bath. The contents were removed and added to 4 ml of scintillant in duplicate. Concentration of 12-DOPP from 0.03 to 3.6 μM were examined in triplicate using platelets from 4 donors.

*Platelet thromboxane generation.* 12-DOPP was added in triplicate to stirred PRP in an aggregometer cuvette to give a final concentration of 0, 0.36, 1.2 and 3.6 μM. Four minutes later 500 μl PRP was removed and added to 50 μl of 100 mM EDTA and 175 μM indomethacin in an Eppendorf tube in an ice bath. Immediately the contents were centrifuged for 3 mins in an Eppendorf centrifuge (15,000 *g*), the plasma was stored at −20° until assayed. The $TxB_2$ content of the plasma was determined by radioimmunoassay (RIA) using a rabbit anti-$TxB_2$ antisera and $^3$H-$TxB_2$ as previously described [17].

*Release of aggregating substance from platelets.* A dose–response curve was obtained for the platelet aggregating activity of 12-DOPP, and the minimum aggregating dose of 0.1 μM was determined. 12-DOPP (1.0 μM) was added to 600 μl of PRP in an aggregometer cuvette and 100 μl PRP were removed at 2 min intervals from 0 to 8 min. The 100 μl of transferred PRP were immediately added to a fresh recipient 600 μl of PRP in an aggregometer cuvette, and the degree of aggregation induced was recorded as before.

*Stability of 12-DOPP in plasma.* High (200 μM) and low (20 μM) concentrations of 12-DOPP in plasma were incubated at 37° for up to 2 hr. Aliquots of 100 μl of plasma were removed at 0, 10, 30, 60 and 120 min. The samples were extracted with methylene chloride and tested for the presence of 12-DOPP and its metabolites by preparative thin-layer chromatography (t.l.c.) combined with direct insertion mass-spectrometry [15].

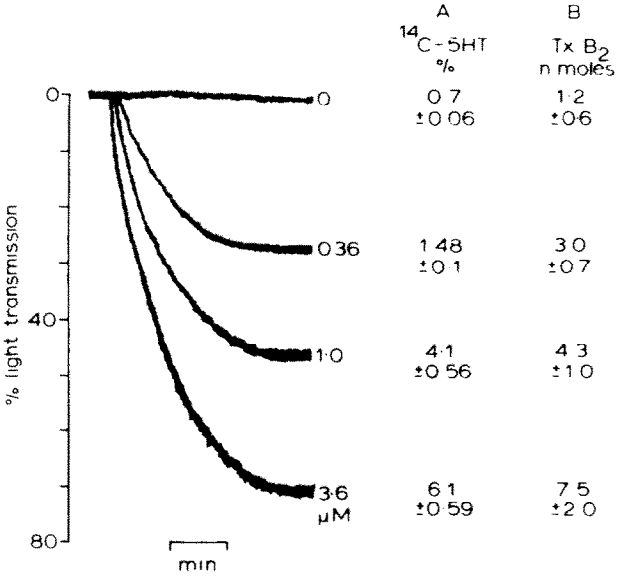


Fig. 1. 12-DOPP induced human platelet aggregation. 12-DOPP was added to give the final concentration shown by each curve. Column A is the mean ± S.E. of the per cent $^{14}$C-5HT release induced by each concentration of 12-DOPP. Column B is the mean ± S.D. of $TxB_2$ (nmoles) generated by each concentration of 12-DOPP.

## RESULTS AND DISCUSSION

12-Deoxyphorbolphenylacetate (12-DOPP), a pro-inflammatory tigliane ester, induced human blood platelet aggregation in the concentration range 0.10–3.6 $\mu$M. Its interaction with platelets has been shown to be structurally specific [8] and it is possible that a 12-DOPP receptor exists on platelets. During aggregation it was found that only between 0.7 and 6.1 per cent (Fig. 1) of $^{14}$C-5HT was released. Also $TxB_2$ generation was low (7.5 nM). This is in contrast to the release reaction and $TxB_2$ generation induced by other aggregating agents such an collagen which generates 60–100 $\mu$M of $TxB_2$ when measured by an identical radio-immune assay method [17].

12-DOPP aggregation is susceptible to inhibition by prostaglandins such as $PGI_2$ and $PGE_1$ [Figs. 2(b) and (c)] which are known to inhibit platelet aggregation by elevation of cyclic-AMP levels [31]. The effect of 12-DOPP on platelets was also shown to be dependent upon bivalent cations since the effect was inhibited by EDTA [Fig. 2(a)]. Furthermore 12-DOPP induced aggregation could be antagonised by colchicine which is known to inhibit the microtubule assembly of platelets [32]. 12-DOPP therefore induces an aggregation of platelets but its mechanism of action differs from that of known aggregating substances.

To investigate the role of arachidonic acid metabolites in 12-DOPP stimulation of platelets we examined the effects of certain inhibitors of thromboxane

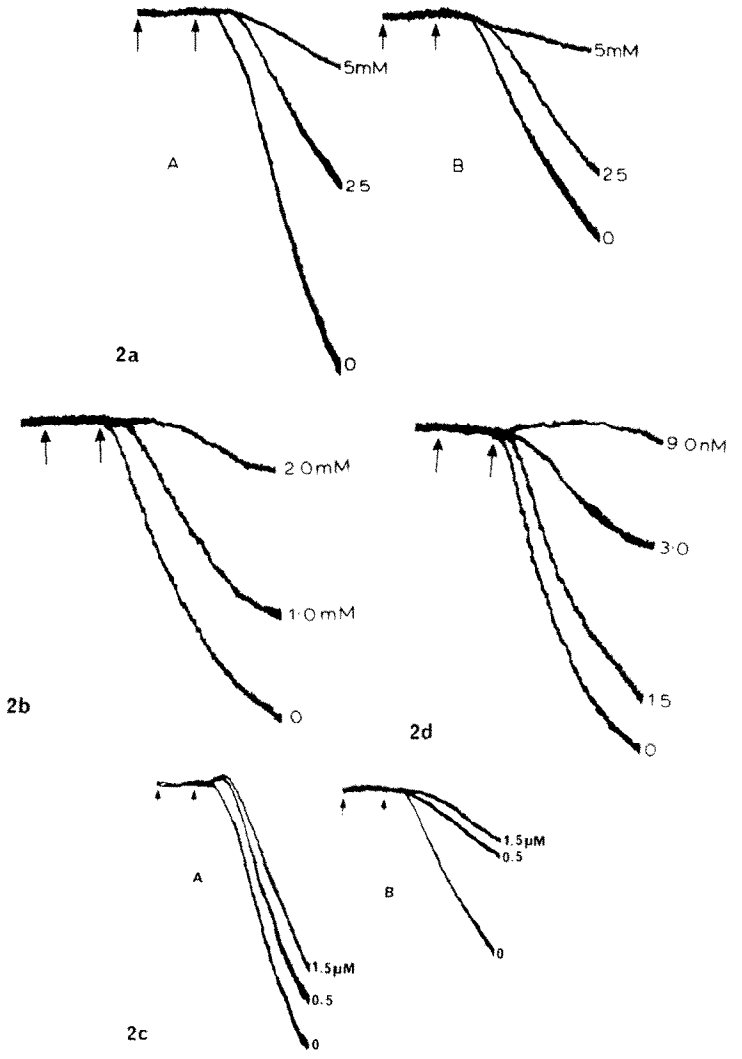


Fig. 2. (a) Effect of EDTA on high (A) and low (B) concentration of 12-DOPP induced human platelet aggregation. EDTA was added at the first arrow to give the final concentration shown by each curve. 12-DOPP was added 1 min later to give a final concentration of A (17.2 $\mu$M) and B (1.29 $\mu$M). (b) Effect of colchicine on 0.86 $\mu$M 12-DOPP induced human platelet aggregation. Colchicine was added at the first arrow 1 min before 12-DOPP to give the final concentration shown by each curve. (c) Effect of $PGE_1$ on high (A) and low (B) concentrations of 12-DOPP induced human platelet aggregation. $PGE_1$ was added at the first arrow to give the final concentration shown by each curve. 12-DOPP was added 1 min later at the second arrow at a concentration of A (17.2 $\mu$M) and B (1.29 $\mu$M). (d) Effect of $PGI_2$ (1.5–9.0 nm) on 1.44 $\mu$M 12-DOPP induced human platelet aggregation. $PGI_2$ was added at the first arrow 1 min before 12-DOPP to give the final concentration shown by each curve.

Table 1. Compounds which had no effect on 12-DOPP induced human platelet aggregation.

| Compound | Concentration (mM) | Action | Reference |
|---|---|---|---|
| Indomethacin | 0.03–0.1 | Cyclo-oxygenase inhibitor | [18] |
| Imidazole | 1–8 | Thromboxane synthetase inhibitor | [19] |
| Clotrimazole | 0.1–0.8 | Thromboxane synthetase inhibitor | [20] |
| 9,11-Aza-prosta-5-13-dienoic acid | 0.02–0.1 | Thromboxane synthetase inhibitor | [21] |
| Pinane thromboxane $A_2$ | 0.001–0.0004 | PG endoperoxide/Tx antagonist | [22] |
| Trimethoquinone | 0.01–0.1 | PG endoperoxide/Tx antagonist | [23] |
| Yohimbine | 0.2–0.5 | α-Antagonist | [24] |
| Verapamil | 0.003–0.3 | Calcium antagonist | [25] |
| Aminopyrine | 0.2–2.0 | Free radical scavengers | [26] |
| Thioanisole | 0.2–2.0 | Free radical scavengers | [26] |
| Butylated hydroxytoluene | 0.07–1.4 | Free radical scavenger and antioxidant | [27] |
| Phenidone | 0.5 | Cyclo-oxygenase and lipoxygenase | [28] |
| FPL 55712 | 0.005–0.06 | Leucotriene B and C antagonist | [29] |

Compounds were examined in concentrations which have been previously shown to have a selective pharmacological action as described by the relevant authors.

synthetase, namely imidazole, clotrimazole and 9,11-aza-prosta-5-13-dienoic acid [20, 21], and antagonists of the thromboxane/PG-endoperoxide receptor, pinane $TxA_2$ [22] and trimethoquinone [23] (Table 1). However we found that none of these substances had any effect on 12-DOPP induced platelet aggregation. In addition aggregation could not be antagonised by indomethacin and it is unlikely that 12-DOPP stimulation of platelets is induced by means of cyclo-oxygenase product release [33]. The tumour-promoting agent TPA has previously been shown to release prostaglandins from MDCK cells [13] and mouse calvaria [12], and although species variations and tissue susceptibilities could account for the differences in the effects of tigliane esters, it has been suggested that esters act at different receptors [11]. The differences in activity between various tigliane esters was further demonstrated *in vivo* by their effects on rabbit skin micro-vasculature where 12-DOPP was found to cause a potent vasoconstriction whereas TPA had no significant effects [43].

The fact that cyclo-oxygenase products are not involved in 12-DOPP aggregation was further confirmed by the observation that the free radical scavengers, aminopyrine, thioanisole and butylated-hydroxy-toluene had no effect upon 12-DOPP platelet aggregation. It has been shown [34] that free radical formation is an initiating step in the biosynthesis of prostaglandins. Furthermore, 12-DOPP induced platelet aggregation is not dependent upon lipoxygenase activity because phenidone, a cyclo-oxygenase and lipoxygenase inhibitor [28] and FPL 55712, a leucotriene B and C antagonist had no effect upon 12-DOPP stimulation of platelets (Table 1). In addition yohimbine, a well-known α-antagonist which inhibits adrenalin induced aggregation, was ineffective in the modification of 12-DOPP induced aggregation.

12-DOPP induces its direct effect upon platelets by interaction at the platelet membrane because the membane stabilising agents imipramine and desmethylimipramine [38] both inhibit the aggregation produced by 12-DOPP [Fig. 3(a)]. It has been suggested that calmodulin may control phospholipase $A_2$ [35], myosin light chain kinase and phosphorylase kinase [36].

Certain phenothiazine derivatives, trifluoperazine being the most potent, have been shown to selectively bind calmodulin and block its interaction with calcium [37]. Thus it was of interest to determine the effect of phenothiazines on 12-DOPP induced aggregation. Trifluoperazine and promethazine were found to be effective inhibitors at concentrations similar to that used by Levin and Weiss [37] and by Mills and Roberts [38] for the inhibition of ADP and adrenalin induced aggregation [Fig. 3(b)]. Accordingly phospholipid mobilisation may play a part in 12-DOPP induced aggregation. Propranolol [39], mepacrine and bromophenacylbromide [40] have been shown to be phospholipase $A_2$ inhibitors. Propranolol is also an inhibitor of 12-DOPP induced aggregation (Fig. 4), and mepacrine at a concentration of 0.21 mM and bromophenacyl-bromide (0.3 mM) produced a 50 per cent inhibition of 12-DOPP induced aggregation. The concentrations used of propranolol and bromophenacyl-bromide were similar to those used previously [39, 40], whereas the mepacrine concentration was ten-fold higher than has previously been used [40]. This evidence indicates that phospholipid mobilisation is involved in 12-DOPP induced platelet aggregation. However, because of the low generation of $TxB_2$ any liberated arachidonate is not metabolised via this pathway. Of the biologically active phospholipid products, lysophosphatidic acids (LPA), in particular palmitoyl-LPA and oleoyl-LPA are effective inducers of human platelet aggregation [41]. Other platelet active phospholipids, known as platelet activating factor (PAF) have also been described [42]. PAF is thought to be 1-*O*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AC-9EPC) [43] a closely related mixture which may represent a series of biologically active lipids. 12-DOPP releases a transferable substance from platelets (Fig. 5) which induces further platelet aggregation. It is possible

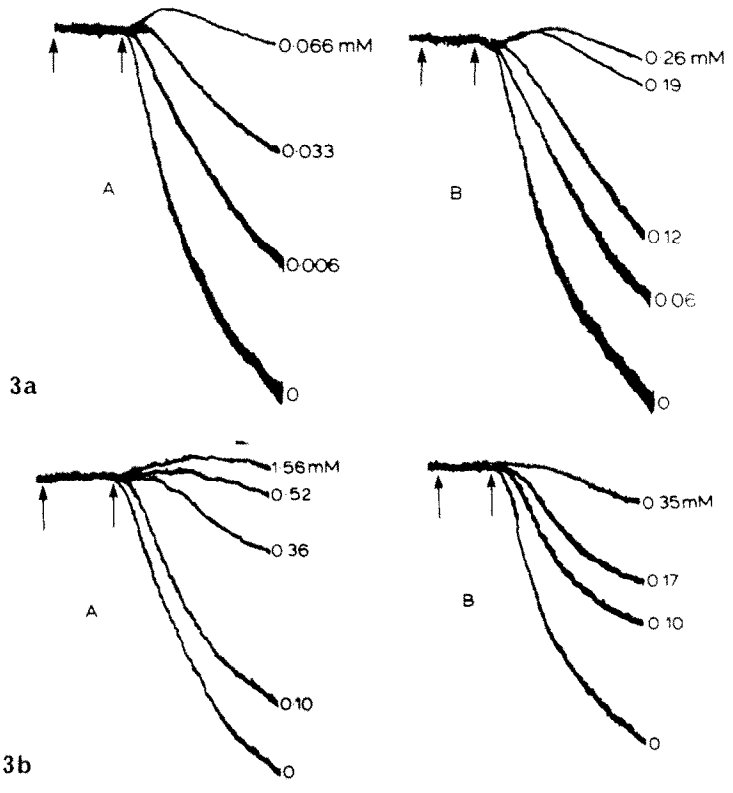


Fig. 3. (a) Effect of desmethylimipramine (A) and imipramine (B) on 1.44 $\mu$M 12-DOPP induced human platelet aggregation. The antagonists were added at the first arrow 1 min before 12-DOPP to give the final concentration shown by each curve. (b) Effect of promethazine (A) (0.006–0.066 mM) and trifluoperazine (B) (0.06–0.26 mM) on 12-DOPP induced human platelet aggregation. The antagonists were added at the first arrow 1 min before 12-DOPP (1.72 $\mu$M) to give the final concentration shown by each curve.

that this factor is related to the PAF class of lipids or maybe this substance represents arachidonic acid which is not converted to thromboxane (Fig. 1). However, it is unlikely to be PAF or AC-9EPC because these lipids induce a 50 per cent release of

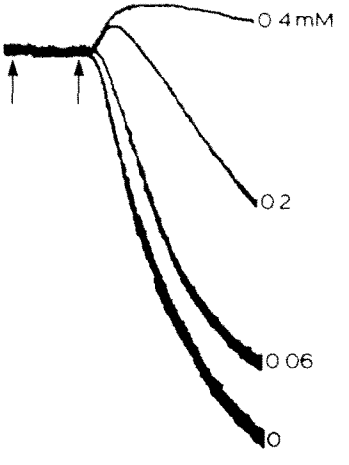


Fig. 4. (a) Effect of propranolol on 1.44 $\mu$M 12-DOPP induced human platelet aggregation. Propranolol was added at the first arrow 1 min before 12-DOPP to give the final concentrations shown by each curve.

5-HT from platelets whereas 12-DOPP induces only a 6 per cent release reaction. Furthermore the importance of PAF as a third pathway of inducing platelet aggregation has not been established [43].

12-DOPP initially has an immediate direct effect upon platelets, inducing agggegation and this activity is structurally specific [8]. This is followed by an indirect effect involving the release of an aggregating substance, maximally 2 min after 12-DOPP induced aggregation (Fig. 5). A chemical analysis of 12-DOPP confirmed that 12-DOPP is not metabolised over this period in human plasma at 37°. 12-DOPP at a concentration of 200 and 20 $\mu$M recovered from plasma by means of methylene chloride partition over a period of 2 hr exhibited only one spot by means of TLC analysis which had the same $R_f$ value as authentic material [15]. Furthermore when the residue from TLC purification was analysed by direct insertion M.S. on an AE1-MS-902 instrument at 210° and 70 e.v. a molecular ion ($M^{+\cdot}$) was observed at $m/z$ 466 (2 per cent, $C_{28}H_{34}C_6$) together with fragment ions at $m/z$ 448 (4 per cent), 430 (4 per cent), 375 (41 per cent), 357 (72 per cent), 330 (31 per cent, $C_{20}H_{26}O_4$), 312 (100 per cent, $C_{20}H_{24}O_3$), 294 (71 per cent). Thus the transferable substance is not a 12-DOPP metabolite or decomposition product. This observation may also be of significance concerning the pro-inflammatory activity of 12-DOPP because

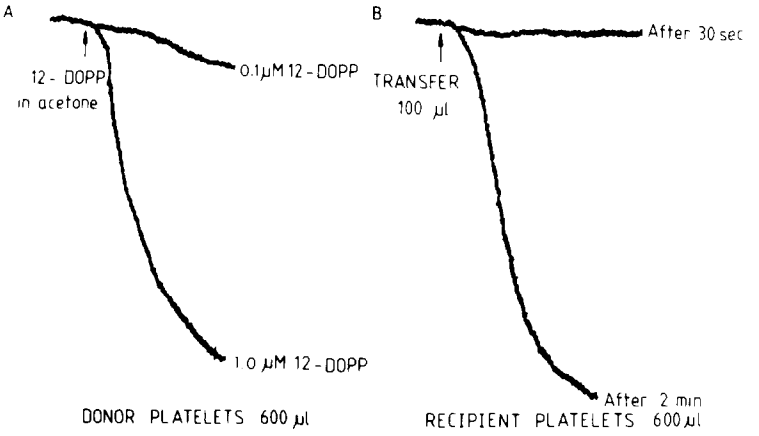


Fig. 5. Induction of human platelet aggregation by 1.0 μM 12-DOPP (A), and the effect of 100 μl of transferred PRP on recipient platelets (B).

it has been shown [44] that induction of inflammation *in vivo* is not the result of direct pharmacological action on the skin micro-vasculature but may be due to the release of mediators [43]. Furthermore it has recently been demonstrated that calmodulin inhibitors such as trifluoperazine and phospholipase $A_2$ inhibitors such as propranolol inhibit 12-DOPP induced erythema in mice skin [45].

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