

# Lack of inhibition of thrombin-induced rise in intracellular $\text{Ca}^{2+}$ levels and 5-hydroxytryptamine secretion by 1-oleoyl-2-acetyl glycerol in human platelets

Sushila Krishnamurthi, Sunil K. Joseph and Vijay V. Kakkar

*Thrombosis Research Unit, King's College School of Medicine & Dentistry, Denmark Hill, London SE5 8RX, England*

Received 28 November 1985

The effect of 1-oleoyl-2-acetyl glycerol (OAG) on the thrombin-induced rise in intracellular  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) and 5-hydroxy $^{14}\text{C}$ tryptamine ( $^{14}\text{C}$ ]5HT) secretion was studied. In washed human platelets pre-labelled with  $^{14}\text{C}$ ]5HT and quin 2, OAG (10–50  $\mu\text{g}/\text{ml}$ ) induced no significant aggregation,  $^{14}\text{C}$ ]5HT secretion or rise in  $[\text{Ca}^{2+}]_i$  in the presence or absence of fibrinogen. However, addition of OAG (10–50  $\mu\text{g}/\text{ml}$ ) 10 s to 5 min before or 10–60 s after addition of threshold concentrations of thrombin ( $<0.03 \text{ U}/\text{ml}$ ) resulted in a significant potentiation of aggregation and  $^{14}\text{C}$ ]5HT secretion without any effect on the thrombin-induced rise in  $[\text{Ca}^{2+}]_i$ . Both EGTA, which abolished the latter and creatine phosphate/creatine phosphokinase, the ADP scavenger, totally inhibited the aggregation but only partially reduced  $^{14}\text{C}$ ]5HT secretion in response to thrombin plus OAG. At higher concentrations of thrombin, neither the rise in  $[\text{Ca}^{2+}]_i$  nor the extent of  $^{14}\text{C}$ ]5HT secretion was significantly altered by OAG addition. The results demonstrate that, unlike phorbol esters, OAG has no inhibitory effect on thrombin-induced  $[\text{Ca}^{2+}]_i$  mobilisation but can synergize with low concentrations of thrombin in potentiating  $^{14}\text{C}$ ]5HT secretion even at basal  $[\text{Ca}^{2+}]_i$ .

*Diacylglycerol (Human platelet) Secretion Thrombin  $\text{Ca}^{2+}$*

## 1. INTRODUCTION

Recent studies have demonstrated that a number of agonist-induced receptor-mediated processes in different cell types can be inhibited by tumour-promoting phorbol esters, which exhibit protein kinase C-activating properties [1–7]. In platelets, it has been shown that phorbol esters such as phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate can inhibit phosphoinositide breakdown, intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) mobilisation and granule secretion induced by agonists such as thrombin, and platelet-activating factor [1–4]. These observations have led to the idea that protein kinase C activation may have a role to play in the termination of agonist-induced transduction processes, which lead to platelet activation [5].

The effects of phorbol esters on protein kinase C are mimicked by diacylglycerol (DAG) [8,9], which is formed endogenously upon agonist stimu-

lation in platelets, as well as in other cells [10,11] via the phosphodiesteratic cleavage of the phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate ( $\text{PtdIns-4,5P}_2$ ). In intact platelets, only certain exogenously added diacylglycerols with optimum water and lipid solubility characteristics enabling them to partition effectively into the lipid bilayer have protein kinase C-activating properties, and these include 1-oleoyl-2-acetyl glycerol (OAG) and 1,2-didecanoylglycerol [12,13]. Although OAG and phorbol esters have similar stimulatory effects on platelet secretion and aggregation [8,14,15] because of their protein kinase C-activating properties, recent work suggests that certain basic differences between these compounds exist regarding their effect on the platelet adenylate cyclase system and the role of endogenously released ADP [16]. This study, which was aimed at further characterisation of the effects of OAG on platelets, demonstrates further

differences in the mechanism of action of phorbol esters and OAG. In our experiments, OAG had no inhibitory effect on the rise in  $[Ca^{2+}]_i$  or 5-hydroxytryptamine (5HT) secretion induced by thrombin, although at low thrombin concentrations and at basal  $[Ca^{2+}]_i$ , synergistic potentiation of 5HT secretion by OAG and thrombin was observed.

## 2. EXPERIMENTAL

Blood collection from apparently healthy human male volunteers, who had denied taking any medication for at least 10 days, and preparation of platelet-rich plasma (PRP) were carried out as described [17]. Washed platelets were prepared by addition of 0.11 N citric acid and 30 nM  $PGI_2$  to PRP, followed by centrifugation of the PRP at  $1500 \times g$  for 15 min. The platelet pellet was resuspended in a pH 6.5 buffer composed of 36 mM citric acid, 5 mM KCl, 103 mM NaCl, 0.5 mM  $CaCl_2$  and 0.09% glucose. For studies on the measurement of 5HT release and  $[Ca^{2+}]_i$ , platelets resuspended in this buffer were incubated with  $0.1 \mu Ci/ml$  of  $[^{14}C]5HT$  (50 Ci/mmol, Amersham) plus  $5 \mu M$  quin 2 acetoxymethyl ester (Amersham) or with  $5 \mu M$  quin 2 ester alone respectively, for 30 min at  $37^\circ C$  in the presence of 0.05 U/ml hirudin. Prior to centrifugation at  $1500 \times g$  for 10 min, 30 nM  $PGI_2$  and 1 U/ml apyrase (grade V, Sigma) were added and following centrifugation, the platelet pellet was resuspended in a pH 7.4 buffer composed of 10 mM Hepes, 103 mM NaCl, 5 mM KCl, 1 mM  $MgSO_4$ , 1 mM  $CaCl_2$  and 0.09% glucose at a platelet count of  $1-1.5 \times 10^8$  or  $2.5-3 \times 10^8/ml$  for the  $[Ca^{2+}]_i$  and  $[^{14}C]5HT$  determinations, respectively. In some experiments, hirudin (0.05 U/ml) was added at the start of the experiment, but this did not significantly affect the results obtained.

OAG (Calbiochem), which was obtained as a solution dissolved in hexane, was dried down under  $N_2$  and re-dissolved in dried dimethyl sulfoxide (DMSO) as a 100 mg/ml solution. In all the experiments with OAG and thrombin, OAG or DMSO vehicle (0.04%) was added 10 s to 10 min before or 10 s to 2 min after thrombin addition. Platelet aggregation was measured as the percentage increase in light transmission as in [17]. For the  $[^{14}C]5HT$  experiments, incubations were termi-

nated 3 min after thrombin addition with EDTA (16 mM) plus formaldehyde (1%), followed by rapid centrifugation of the samples and collection of the supernatants for  $^{14}C$  liquid scintillation counting. For the studies on  $[Ca^{2+}]_i$  determinations, fluorescence measurements were carried out in quin 2-loaded platelets incubated at  $37^\circ C$  in a Perkin Elmer LS-5 luminescence spectrometer. Calibration of the fluorescence signals and calculation of  $[Ca^{2+}]_i$  (in nM) were carried out as described by Tsien et al. [18]. In some experiments, 5 mM EGTA was added 2 min before addition of OAG and thrombin to enable  $[^{14}C]5HT$  and  $[Ca^{2+}]_i$  determinations to be made in the absence of extracellular  $Ca^{2+}$ . Statistical analysis of all the data was carried out using the Student's *t*-test for unpaired data.

## 3. RESULTS

Addition of OAG (10–50  $\mu g/ml$ ) to quin 2/ $[^{14}C]5HT$ -labelled washed platelets in the presence or absence of 0.5–1 mg/ml fibrinogen (human, Kabi), resulted in no significant aggregation (fig.1),  $[^{14}C]5HT$  release or rise in  $[Ca^{2+}]_i$  (fig.2) compared with that in resting, unstimulated platelets. This was found to be the case even when OAG from another source (Nova Biochem) was tested (not shown). However, OAG (10–50  $\mu g/ml$ ) was able to potentiate significantly both the aggregation and  $[^{14}C]5HT$  secretion, when added 10 s to 5 min before or 10–60 s after addition of sub-threshold to threshold concentrations of thrombin (0.01–0.03 U/ml), which induced little or no aggregation and  $[^{14}C]5HT$  release on their own (figs 1,2). The data in figs 1 and 2 are those obtained in indomethacin-treated platelets, although the absence of indomethacin pre-treatment did not affect any of the results obtained (not shown). The small rise in  $[Ca^{2+}]_i$  induced by these concentrations of thrombin, ranging between 100 and 200 nM in the presence of 1 mM external  $Ca^{2+}$ , was not significantly affected ( $p > 0.05$ ) by addition of OAG (fig.2). The potentiation of thrombin (0.01–0.03 U/ml)-induced  $[^{14}C]5HT$  secretion by OAG was still present in EGTA-treated platelets, in which no significant aggregation (not shown),  $[^{14}C]5HT$  secretion or rise in  $[Ca^{2+}]_i$  in response to thrombin alone was noted (fig.2). However, the extent of  $[^{14}C]5HT$  release in response to OAG + thrombin was signifi-

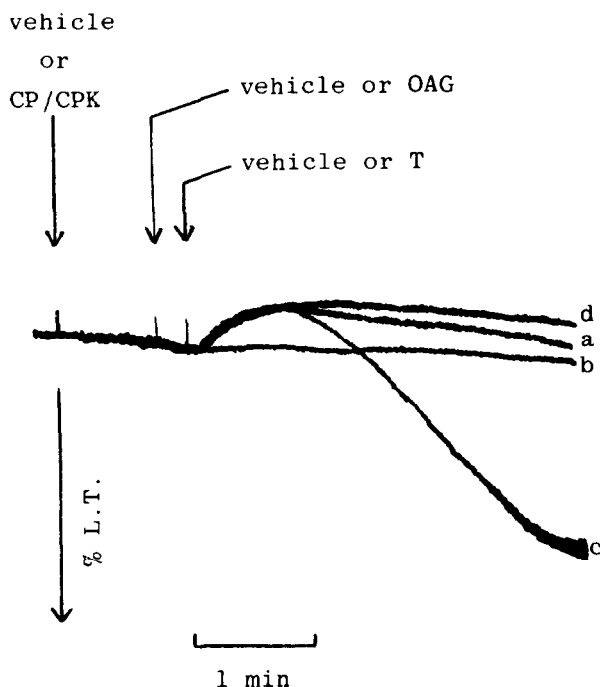


Fig.1. Effect of OAG on threshold thrombin (0.02 U/ml)-induced human platelet aggregation in the presence of 1 mM external  $\text{Ca}^{2+}$ . Indomethacin ( $10\mu\text{M}$ ) and imipramine ( $3\mu\text{M}$ ) treated platelets pre-loaded with quin 2 were pre-incubated with vehicle or CP/CPK for 1 min at  $37^\circ\text{C}$  prior to vehicle or OAG ( $25\mu\text{g/ml}$ ) addition, followed 10 s later by thrombin (T). Traces a-d represent vehicle + T, OAG + vehicle, OAG + T and CP/CPK + OAG + T, respectively. For trace b, fibrinogen ( $0.5\text{--}1\text{ mg/ml}$ ) was present. The traces shown are typical of 3 similar experiments using platelets from different donors.

cantly ( $p < 0.005$ ) smaller in EGTA-treated platelets ( $19.6 \pm 4.4\%$ ) than that obtained in the presence of 1 mM external  $\text{Ca}^{2+}$  ( $37.4 \pm 3.6\%$ ). Similarly, 5 mM creatine phosphate (CP) plus 25 U/ml creatine phosphokinase (CPK), which abolished platelet responses to  $10\mu\text{M}$  ADP (not shown), abolished the potentiation of thrombin-induced aggregation by OAG (fig.1) but only partially reduced the potentiation of [ $^{14}\text{C}$ ]5HT secretion by OAG (fig.2). The extent of [ $^{14}\text{C}$ ]5HT release in platelets treated with CP/CPK plus OAG ( $25\mu\text{g/ml}$ ) plus thrombin was  $10.6 \pm 2.4\%$  and, while this was significantly

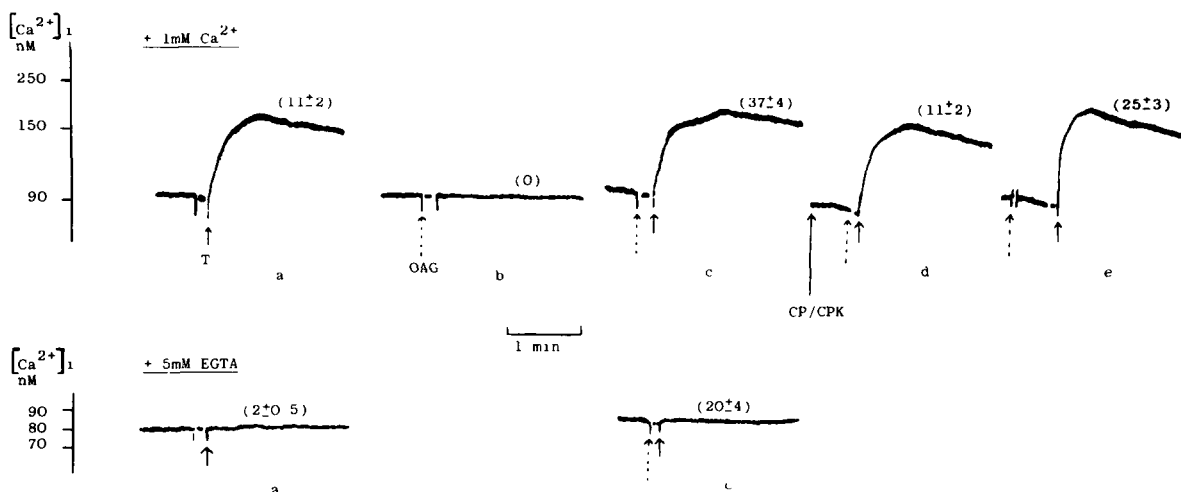


Fig.2. Effect of OAG on threshold thrombin (T) (0.02 U/ml)-induced  $[\text{Ca}^{2+}]_i$  mobilisation and [ $^{14}\text{C}$ ]5HT release in quin 2 and [ $^{14}\text{C}$ ]5HT-labelled platelets, in the presence of 1 mM external  $\text{Ca}^{2+}$  (upper) or 5 mM EGTA (lower). Platelets were pre-treated with indomethacin ( $10\mu\text{M}$ ) and imipramine ( $3\mu\text{M}$ ). Traces a-d are as described in the legend to fig.1 while trace e represents the addition of OAG 5 min before thrombin. The values in parentheses beside each trace denote the percentage [ $^{14}\text{C}$ ]5HT released into the platelet supernatants and represent the means  $\pm$  SE of 9-12 determinations from 3 different donors. The fluorescence traces are representative ones from the same number of experiments. The  $[\text{Ca}^{2+}]_i$  calibration scale is shown along the vertical axis on the left.

Table 1

Effect of OAG on high-dose thrombin-induced rise in  $[Ca^{2+}]_i$  and  $[^{14}C]5HT$  secretion in the presence of 1 mM external  $Ca^{2+}$

	$[Ca^{2+}]_i$ (nM)	% $[^{14}C]5HT$ release
Resting	84 ± 3	0
Vehicle + 0.2 U/ml thrombin	772 ± 177	72 ± 3
OAG + 0.2 U/ml thrombin	596 ± 145	71 ± 3
CP/CPK + OAG + 0.2 U/ml thrombin	610 ± 115	68 ± 1.5

Vehicle or OAG (25 µg/ml) was added 5 min before thrombin in these experiments but essentially similar results were obtained with 10 s to 10 min pre-incubations of vehicle or OAG

( $p < 0.001$ ) less than that obtained in the absence of CP/CPK, it was nevertheless significantly ( $p < 0.02$ ) greater than the extent of  $[^{14}C]5HT$  release obtained with thrombin alone in the presence of CP/CPK, which equalled  $3.8 \pm 2\%$ . The potentiation of thrombin-induced  $[^{14}C]5HT$  secretion by OAG was significant when OAG was added 10–60 s after thrombin or with 10 s to 5 min pre-incubations of OAG but became negligible with 10 min pre-incubations of OAG (not shown). In platelets not loaded with quin 2 the thrombin dose-response curve for  $[^{14}C]5HT$  secretion was shifted approx. 2-fold to the left and potentiatory effects of OAG were observed at approx. 2-fold lower concentrations of thrombin compared to those described in figs 1 and 2.

At higher thrombin concentrations ( $>0.1$  U/ml), where the rise in  $[Ca^{2+}]_i$  and the extent of  $[^{14}C]5HT$  secretion with thrombin alone were significantly higher, OAG added 10 s to 10 min before or 10–60 s after thrombin had no significant ( $p > 0.05$ ) effect on the rise in  $[Ca^{2+}]_i$  or  $[^{14}C]5HT$  secretion induced by thrombin (results with 5 min OAG pre-incubations in table 1). Addition of CP/CPK before addition of OAG and thrombin also did not affect the thrombin-induced rise in  $[Ca^{2+}]_i$  or  $[^{14}C]5HT$  secretion (table 1).

#### 4. DISCUSSION

Although earlier workers [9,14,15] using OAG have demonstrated a small but significant aggrega-

tory and secretory response to OAG, we have been unable to demonstrate any significant aggregatory or secretory response to OAG in our washed platelet preparations, even in the presence of extracellular  $Ca^{2+}$  and fibrinogen. This could be due to the absence of released ADP in our final washed platelet suspensions achieved via the use of apyrase in our washing procedures, as a recent report [16] has demonstrated a critical role for ADP in OAG-induced 40 kDa phosphorylation and aggregation. Interestingly, Lapetina et al. [13] have demonstrated that in platelets isolated in the absence of prostacyclin and resuspended in EGTA-free buffer, OAG and other active analogues including dihexanoyl- and dioctanoylglycerol induced a small aggregation, but when platelets were isolated in the presence of prostacyclin, no aggregation was observed. Taking these findings together, it seems likely that exogenously added diacylglycerols, which can activate protein kinase C in intact platelets, have little or no ability to induce aggregation or secretion on their own but, as earlier work [16] and the present results show, they can synergize strongly with other agonists such as ADP and thrombin in potentiating aggregation and secretion. Synergism between OAG and  $Ca^{2+}$  [8,9,14], which has been described earlier with the use of  $Ca^{2+}$  ionophores, may account, at least partly, for the synergism observed between OAG and low concentrations of thrombin, which induced a small but significant rise in  $[Ca^{2+}]_i$ . However, it is noteworthy that this synergism was also observed in EGTA-treated platelets in the absence of a rise in  $[Ca^{2+}]_i$ , as detected using quin 2 methodology, and could be mediated via fusogenic effects of OAG facilitating secretion [19] or could represent an as yet undescribed mechanism for the potentiation of platelet secretion. Interestingly, aggregation but not  $[^{14}C]5HT$  secretion induced by OAG and low concentrations of thrombin was abolished by CP/CPK indicating that the latter is at least partially independent of released ADP. The role of endogenously formed thromboxane  $A_2$  in these aggregatory or secretory responses can be ruled out as indomethacin, the cyclooxygenase inhibitor, had no significant effect on either phenomenon.

It is significant that, apart from the potentiatory effects described above, OAG had no inhibitory effect on thrombin-induced rise in  $[Ca^{2+}]_i$  at both low and high thrombin concentrations and this

represents a major divergence in the effects of OAG and phorbol esters, which have been shown to inhibit agonist-induced  $[Ca^{2+}]_i$  mobilisation in platelets [1,2]. Furthermore OAG had no significant effect on high-dose thrombin-induced  $[^{14}C]5HT$  secretion and this is at variance with the results of Watson and Lapetina [3], who have recently demonstrated a significant inhibitory effect of OAG on the same. The reasons for this are not clear but one possibility is the presence of small amounts of prostacyclin because of their platelet isolation procedures, and synergistic increases in cyclic AMP levels by OAG and prostacyclin [16] causing inhibition of platelet activation. It is interesting however, that these workers [3], who carried out pre-incubations of OAG with a single time point of 10 min, also failed to demonstrate a potentiatory effect of OAG at low thrombin concentrations, as we did with the 10 min pre-incubations.

This disappearance of the potentiatory effects of OAG on thrombin-induced aggregation and secretion with pre-incubation times greater than 5 min could be due to phosphorylation of OAG into the corresponding phosphatidic acid derivative, which has been shown to occur within 2 min of OAG addition to platelets [9,10,21]. However, the presence of the potentiatory effects even when OAG was added 10–60 s after thrombin further emphasises the important role of endogenously formed DAG in the amplification of agonist-induced platelet responses [8] and, stresses the need for a re-examination of the concept that DAG and/or protein kinase C may have a specific role in the termination of platelet responses.

## REFERENCES

- [1] MacIntyre, D.E., McNicol, A. and Drummond, A.H. (1985) *FEBS Lett.* 180, 160–164.
- [2] Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3859–3862.
- [3] Watson, S.P. and Lapetina, E.G. (1985) *Proc. Natl. Acad. Sci. USA* 82, 2623–2626.
- [4] Rittenhouse, S.E. and Sasson, J.P. (1985) *J. Biol. Chem.* 260, 8657–8660.
- [5] Drummond, A.H. and MacIntyre, D.E. (1985) *Trends Pharmacol. Sci.* 6, 233–234.
- [6] Naccache, P.M., Molski, M.M., Volpi, M., Becker, E.L. and Sha'afi, R.I. (1985) *Biochem. Biophys. Res. Commun.* 130, 677–684.
- [7] Lynch, C.J., Charest, R., Bocckino, S.B., Exton, J.H. and Blackmore, P.F. (1985) *J. Biol. Chem.* 260, 2844–2851.
- [8] Nishizuka, Y. (1984) *Nature* 308, 693–698.
- [9] Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, T.K. and Nishizuka, Y. (1983) *J. Biol. Chem.* 258, 6701–6704.
- [10] Berridge, M.J. (1984) *Biochem. J.* 220, 345–360.
- [11] Hokin, L. (1985) *Annu. Rev. Biochem.* 54, 205–235.
- [12] Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) *J. Biol. Chem.* 260, 5315–5322.
- [13] Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) *J. Biol. Chem.* 260, 1358–1361.
- [14] Rink, T.J., Sanchez, A. and Hallam, T.J. (1983) *Nature* 305, 317–319.
- [15] Knight, D.E. and Scrutton, M.C. (1984) *Nature* 309, 66–68.
- [16] Ashby, B., Kowalska, M.A., Wernick, E., Rigmaiden, M., Daniel, J.L. and Smith, J.B. (1985) *J. Cyclic Nucleotide Protein Phosphorylation Res.* 10, 473–483.
- [17] Krishnamurthi, S., Westwick, J. and Kakkar, V.V. (1982) *Thromb. Haemost.* 48, 136–141.
- [18] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) *J. Cell Biol.* 94, 325–333.
- [19] Kajikawa, N., Kaibuchi, K., Natsubara, T., Kikkawa, U., Takai, Y. and Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* 116, 743–750.
- [20] Watson, S.P., Ganong, B.R., Bell, R.M. and Lapetina, E.G. (1984) *Biochem. Biophys. Res. Commun.* 121, 386–391.
- [21] De Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1984) *Biochem. Biophys. Res. Commun.* 123, 589–595.