Synergistic potentiation of 5-hydroxytryptamine secretion by platelet agonists and phorbol myristate acetate despite inhibition of agonist-induced arachidonate/thromboxane and β -thromboglobulin release and Ca^{2+} mobilization by phorbol myristate acetate

Sushila KRISHNAMURTHI, Sunil JOSEPH and Vijay V. KAKKAR

Thrombosis Research Unit, King's College School of Medicine and Dentistry, Denmark Hill, London SE5 8RX, U.K.

Previous studies have demonstrated an inhibition of agonist-induced inositol phospholipid breakdown and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) mobilization by phorbol esters in platelets. In this study, we have examined the effect of phorbol 12-myristate 13-acetate (PMA) on agonist-induced granule secretion and correlated it with agonist-induced $[\text{Ca}^{2+}]_i$ mobilization, arachidonate and thromboxane (Tx) release in human platelets. With increasing times of incubation with PMA (10 s–5 min), the rise in $[\text{Ca}^{2+}]_i$ induced by thrombin and the TxA_2 mimetic, U46619, was increasingly inhibited (90–100% with 5 min incubation) and, correlating with this, thrombin-induced $[\text{H}^3]$ arachidonate, TxB_2 and β -thromboglobulin (βTG) release were also inhibited. In addition, the conversion of exogenously added arachidonate to TxB_2 was inhibited (50–80%) by a 10 s–5 min pretreatment with PMA. However, secretion of 5-hydroxy ^{14}C tryptamine (5HT) induced by thrombin or U46619 was not inhibited by 10 s–2 min incubations with PMA and, on the contrary, with low agonist concentrations, was potentiated by PMA in the absence of a significant rise in $[\text{Ca}^{2+}]_i$ or endogenous Tx formation, to levels significantly greater than or equal to the sum of that obtained when agonist and PMA were added separately. With longer times of incubation with PMA (5 min), these synergistic effects became less pronounced as inhibitory effects of PMA on agonist-induced ^{14}C 5HT secretion became apparent. The results indicate that, while PMA may cause an inhibition of agonist-induced $[\text{Ca}^{2+}]_i$ mobilization resulting in an inhibition of agonist-induced arachidonate, TxB_2 and βTG release, its effects on agonist-induced 5HT secretion may be complicated by $[\text{Ca}^{2+}]_i$ -independent synergistic effects of agonist and PMA.

INTRODUCTION

Agonist binding to platelet receptors has been shown to result in a number of biochemical changes, the earliest of these being a rapid elevation of $[\text{Ca}^{2+}]_i$ levels and the phosphodiesterase cleavage of $\text{PtdIns}(4,5)\text{P}_2$ by the enzyme phospholipase C (for reviews see [1] and [2]). The latter reaction results in the formation of DAG and $\text{Ins}(1,4,5)\text{P}_3$, both of which appear to play important roles in various cellular activation processes. $\text{Ins}(1,4,5)\text{P}_3$ has been implicated in the release of Ca^{2+} from intracellular stores in various cells (reviewed in [3]) including platelets [4,5] while DAG, which activates the enzyme protein kinase C and causes the phosphorylation of a 40–47 kDa protein [6], can promote platelet secretion even at basal Ca^{2+} levels [7,8]. The activation of protein kinase C by DAG can be mimicked by tumour-promoting phorbol esters such as PMA and phorbol 12,13-dibutyrate [9], which also induce platelet secretion at basal $[\text{Ca}^{2+}]_i$ levels [8], although secretion induced by all these agents can be greatly potentiated by small increases in Ca^{2+} [6–8].

However, in addition to the stimulatory effects on platelets, and other cells, several workers [10–13] have reported inhibitory effects of phorbol esters on various intracellular processes activated by other agonists. In platelets, it has been reported that phorbol esters can cause an inhibition of $\text{PtdIns}(4,5)\text{P}_2$ breakdown, phosphatidate synthesis, $[\text{Ca}^{2+}]_i$ mobilization and granule secretion induced by agents such as thrombin, platelet-activating factor and vasopressin [14–17]. During the course of a series of studies on agonist interactions and 5HT secretion, we have observed positive synergistic effects between PMA and agonists such as ADP, platelet-activating factor and the thromboxane A_2 mimetic, U46619 (11 α -epoxy-9 α -methanoprostaglandin H_2) [18] in platelets pretreated with EGTA or indomethacin, resulting in enhanced 5HT secretion in the absence of a significant rise in $[\text{Ca}^{2+}]_i$ or endogenous thromboxane formation [19]. As these results were clearly not in agreement with the earlier reports, we have carried out a detailed study on the effects of PMA on 5HT secretion induced by thrombin and U46619 and correlated it with its effects on $[\text{Ca}^{2+}]_i$ mobilization, arachidonate release

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} ; 5HT, 5-hydroxytryptamine; Tx, thromboxane; PMA, phorbol 12-myristate 13-acetate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; PRP, platelet-rich plasma; βTG , β -thromboglobulin.

and thromboxane synthesis induced by these agonists, varying both agonist concentration and incubation time with PMA.

MATERIALS AND METHODS

Materials

PMA, 4 β -phorbol (Sigma) and Quin 2 acetoxymethyl tetraester (Amersham International) were dissolved in dimethyl sulphoxide. Thrombin (bovine; Parke Davis) was made up in iso-osmotic saline. U46619, which was kindly given by Dr. J. Pike, Upjohn Company, Kalamazoo, MI, U.S.A., was dissolved in ethanol, and diluted in iso-osmotic saline to give working solutions of the desired concentration. Sodium arachidonate (Sigma) and indomethacin (Sigma) were dissolved in ethanol, while phenidone (Sigma) was dissolved in dimethyl sulphoxide/ethanol (1:1, v/v).

Measurement of [14 C]5HT, [3 H]arachidonate, TxB $_2$ and β TG in platelet supernatants

Blood collection from healthy male volunteers, who had denied taking any medication for at least 10 days, preparation of platelet-rich plasma (PRP) and washed platelets, studies on dense-granule release in platelets prelabelled with [14 C]5HT (57 Ci/mol; Amersham International) and measurement of TxB $_2$ in platelet supernatants by radioimmunoassay were done as previously described [20]. The release of β TG, a platelet α -granule marker [21], into the supernatant of stimulated platelets was measured by using radioimmunoassay kits from Amersham International.

Thrombin-induced release of [14 C]5HT, TxB $_2$ and β TG was studied in washed platelets resuspended in a pH 7.4 buffer (buffer B) composed of 10 mM-Hepes, 103 mM-NaCl, 5 mM-KCl, 1 mM-MgSO $_4$, 1 mM-CaCl $_2$, 0.35% bovine serum albumin, 0.09% glucose and 0.05 unit of hirudin/ml [platelet count (2.5–3.0) $\times 10^8$ /ml] while U46619- and sodium arachidonate-induced release were studied using PRP [platelet count (2.5–3.0) $\times 10^8$ /ml] as the results were more consistent than in washed platelets. The range of PMA concentrations tested in PRP and washed platelets was 160–1600 nM and 1–160 nM respectively.

For studies on [3 H]arachidonate release, platelets in a pH 6.5 buffer (buffer A), composed of 36 mM-citric acid, 5 mM-KCl, 103 mM-NaCl, 0.5 mM-CaCl $_2$, 0.35% bovine serum albumin and 0.09% glucose, were incubated with 0.5 μ Ci of [3 H]arachidonic acid/ml (100 Ci/mmol; Amersham International) for 90 min at 37 °C, in the presence of hirudin (0.05 unit/ml). Prior to centrifugation at 1500 *g* for 15 min, prostaglandin I $_2$ (30 nM) was added to the platelets and following centrifugation, the platelet pellet was resuspended in buffer B to a platelet count of (4–5) $\times 10^8$ /ml. Metabolism of the released arachidonate via the cyclo-oxygenase and lipoxygenase pathways was prevented in these platelets by the addition of 500 μ M-phenidone [22].

The effect of PMA on agonist-induced release of [3 H]arachidonate, [14 C]5HT, TxB $_2$ and β TG, which was carried out in stirred platelets at 37 °C, was studied by the addition of PMA or dimethyl sulphoxide vehicle (0.04%) 10 s–5 min before agonist addition. This was compared with experiments where PMA was added to platelets at the indicated times and vehicle (iso-osmotic

saline) was substituted for agonist. In all cases, the reactions were terminated 180 s after addition of agonist or vehicle with EDTA (16 mM) plus formaldehyde (1%) after which the platelet suspensions were rapidly centrifuged and the supernatants collected for measurement of the various markers. Initial experiments on the time-course of thrombin-induced [14 C]5HT release, and on the effect of pretreating platelets with imipramine (5–20 μ M), revealed that no significant re-uptake of [14 C]5HT by the platelets occurred over a 3–15 min period after thrombin addition, and further experiments were therefore conducted without pretreatment of the platelets with imipramine. The release of [3 H]arachidonate was quantified by counting the supernatants as for the [14 C]5HT studies, and expressing the released label as a percentage of the total radioactivity incorporated into the platelets. In initial experiments, these supernatants were subjected to organic extraction followed by t.l.c. of the dried extracts [23], and these studies revealed that all the released label co-migrated with authentic arachidonic acid.

Measurement of [Ca^{2+}] $_i$ levels

These were carried out by incubating platelets resuspended in buffer A (minus albumin) with 7 μ M-Quin 2 ester for 30 min at 37 °C, in the presence of hirudin (0.05 unit/ml). Following the incubation period, centrifugation and resuspension of the platelets in buffer B (minus albumin) at a platelet count of (1.0–1.5) $\times 10^8$ /ml was carried out as described above for the [3 H]arachidonate studies. Fluorescence measurements at 37 °C were carried out with a Perkin-Elmer LS5 luminescence spectrometer, with excitation at 339 nm and emission at 500 nm. The cuvette containing the platelets was not stirred and the calculation of the [Ca^{2+}] $_i$ concentration (nM) was carried out as described previously [24].

Statistical analysis

Analysis of the data was carried out by using Student's *t* test for unpaired data.

RESULTS

Effect of PMA on thrombin- and U46619-induced rise in [Ca^{2+}] $_i$ levels, arachidonate and thromboxane release and granule secretion

The effect of PMA on thrombin- and U46619-induced platelet activation depended on the particular phenomenon being examined, and on the incubation time with PMA. At least with [14 C]5HT secretion, the effects obtained with incubation times of 10 s–2 min were distinct from those obtained with longer incubation times of 3–5 min; hence the effects obtained with the short and long incubation times will be dealt with separately.

Preincubation of PMA for 10 s–2 min

Although initial experiments with PMA and its effect on thrombin-induced rise in [Ca^{2+}] $_i$ levels were carried out using a range of PMA concentrations between 1 and 160 nM, all subsequent experiments described in Figs. 1–3 and Tables 1 and 2 were carried out with a single fixed concentration of PMA, which was 16 nM in washed platelets and 400 nM in PRP. These concentrations were found to be maximally effective at inducing platelet

Fig. 1. Effect of PMA (16 nM) on the rise in $[Ca^{2+}]_i$ induced by thrombin (0.02 and 0.2 unit/ml) and U46619 (0.6 and 3 μ M) in Quin 2-loaded platelets resuspended in Hepes-buffered saline (pH 7.4) containing 1 mM- Ca^{2+}

The vertical axis represents the $[Ca^{2+}]_i$ concentration in nM and the mean $[Ca^{2+}]_i$ value calculated from 9–12 determinations in three similarly performed experiments (using platelets from different donors) is shown on each fluorescence trace, which is a representative one. The traces marked 'Control' represent responses obtained with thrombin (T) or U46619 (U) in the absence of PMA but in the presence of dimethyl sulphoxide (0.04%) vehicle added 10 s before either agonist, while the traces marked 'a' and 'b' represent responses obtained in the presence of PMA, added 10 s or 5 min respectively before thrombin or U46619.

* $P < 0.001$ when compared with respective control.

aggregation and $[^3H]5HT$ secretion on their own in the respective media, and are within the range of concentrations that have shown to be effective at activating protein kinase C [6]. Addition of PMA (1–160 nM) to Quin 2-loaded washed platelets had no significant effect on the basal $[Ca^{2+}]_i$ levels (results not shown) which equalled 100 ± 2 nM (mean $\pm$ S.E.M.). However, incubation with 16 nM-PMA for 10 s before addition of thrombin or U46619 resulted in a reduction in the peak $[Ca^{2+}]_i$ level, compared with that obtained in the presence of dimethyl sulphoxide vehicle (Fig. 1). Although there was a definite tendency for the peak $[Ca^{2+}]_i$ to be reduced in the presence of PMA with all concentrations of thrombin and U46619 tested, the reduction with 0.02 unit of thrombin/ml alone, the lowest thrombin concentration tested, was statistically significant ($P < 0.001$). Correlating with the inhibition of $[Ca^{2+}]_i$ mobilization, the TxB_2 generated in response to thrombin (0.02 unit/ml) was also significantly ($P < 0.001$) reduced by a 10 s pretreatment with PMA (Table 1). At a higher thrombin concentration of 0.2 unit/ml, a good correlation between inhibition of $[Ca^{2+}]_i$ mobilization and release of

$[^3H]arachidonate$ and TxB_2 by a 10 s pretreatment with PMA was evident, with the inhibition of the three phenomena being 28%, 22% and 18% respectively. Neither U46619 nor PMA on their own caused any significant release of $[^3H]arachidonate$ or TxB_2 (results with U46619 not shown).

The effect of PMA on thrombin- and U46619-induced β TG release was complicated by the finding that PMA caused large amounts of β TG release, equalling 44% of total, and not significantly different ($P > 0.05$) from that observed with 0.02 unit of thrombin/ml (Table 1) and 0.6–3 μ M-U46619 (results not shown). With these agonist concentrations, therefore, addition of PMA 10 s before the agonist resulted in a similar level of β TG release to that seen with agonists or PMA alone (results with thrombin at 0.02 unit/ml and PMA in Table 1). At a thrombin concentration of 0.2 unit/ml, a significantly greater ($P < 0.02$) level of β TG release (63%) was observed, and this was partially reduced by a 10 s pretreatment with PMA (Table 1).

In contrast with the effects described above, PMA had no inhibitory effect on thrombin- or U46619-induced

Table 1. Effect of PMA (16 nM) on thrombin-induced release of [³H]arachidonate, TxB₂ and β TG

All experiments were carried out in washed platelets resuspended in Hepes-buffered saline (pH 7.4) containing 1 mM-Ca²⁺, at a platelet count of (3–5) × 10⁸/ml. For the thrombin experiments, PMA or vehicle was added 10 s or 5 min before thrombin as indicated and, for the experiments with PMA alone, PMA was added 10 s or 5 min before vehicle, which substituted for thrombin. The values represent means ± S.E.M. for data (9–12 determinations) from three similarly performed experiments, using platelets from different donors. ***P* < 0.002, ****P* < 0.001 compared with vehicle plus thrombin.

Pretreatment with PMA or vehicle	Thrombin (unit/ml)	Release of:		
		[³ H]Arachidonate (% of total c.p.m.)	TxB ₂ (pmol/10 ⁸ platelets)	β TG† (% release)
Resting	–	2.1 ± 0.07	1.6 ± 1.0	0.55 ± 0.03
PMA (10 s)	Vehicle	–	0.9 ± 0.2	44 ± 3
PMA (5 min)	Vehicle	2.5 ± 0.12	3 ± 1	44 ± 3
Vehicle (10 s)	0.02	–	107 ± 25	37 ± 2
PMA (10 s)	0.02	–	14 ± 5 ***	34 ± 2
PMA (5 min)	0.02	–	3 ± 1 ***	40 ± 2
Vehicle (10 s)	0.2	11.2 ± 0.10	342 ± 35	63 ± 5
PMA (10 s)	0.2	9.2 ± 0.13***	281 ± 30	50 ± 2
PMA (5 min)	0.2	6.6 ± 0.80***	89 ± 19**	40 ± 2**

† The total amount of β TG measurable after repeated freeze–thawing of the platelets was 29 ± 2.7 μ g/10⁹ platelets.

Fig. 2. Effect of PMA (16 nM) on thrombin-induced [¹⁴C]5HT secretion in washed platelets resuspended in Hepes-buffered saline (pH 7.4) containing 1 mM-Ca²⁺

The curves marked 'Control', 'a', 'b' and 'c' represent responses to thrombin in the absence of PMA, and in platelets pretreated with PMA for 10 s, 5 min and 2 min respectively. Dimethyl sulphoxide (0.04%), was added 10 s before thrombin for the control incubations. Preincubation with dimethyl sulphoxide for up to 5 min before thrombin addition did not alter the results obtained. The responses obtained with PMA alone are represented as the results obtained in the absence of thrombin. The broken line represents the effect of PMA, added 10 s before thrombin in indomethacin (5 μ M)-treated platelets. The data represented are means ± S.E.M. of 9–12 determinations from three similarly performed experiments, using platelets from different donors. **P* < 0.002 and ***P* < 0.01 respectively compared with respective controls.

Fig. 3. Effect of PMA (400 nM) on U46619-induced [¹⁴C]5HT secretion in PRP

◇, The effect of PMA added 10 s before U46619 or vehicle in EGTA (5 mM)-treated platelets; ◆, the response to U46619 alone in EGTA-treated platelets. All other conditions are as described in Fig. 2.

[¹⁴C]5HT secretion when added 10 s–2 min before either agonist. On the other hand, the [¹⁴C]5HT dose–response curves to thrombin and U46619 were shifted up to 4-fold to the left, depending on agonist concentration, in the presence of PMA (Figs. 2 and 3). With subthreshold to threshold concentrations of thrombin and U46619, PMA caused a significant potentiation of agonist-induced [¹⁴C]5HT secretion, which was significantly (*P* < 0.02) greater than the sum of the percentage release obtained

when PMA and agonist were added alone (Figs. 2 and 3), e.g. PMA alone, $12\% \pm 4\%$; 0.01 unit of thrombin/ml alone, $6\% \pm 4\%$, PMA + 0.01 unit of thrombin/ml, $44\% \pm 4\%$, when PMA was added 10 s before thrombin (Fig. 2). With higher concentrations of thrombin and U46619, which induced 20–50% [^{14}C]5HT release on their own, addition of PMA 10 s–2 min before the agonist resulted in a simple additive effect of PMA and agonist on [^{14}C]5HT secretion, e.g. PMA alone, $16\% \pm 2\%$, $3\text{ }\mu\text{M}$ -U46619 alone, $49\% \pm 3\%$; PMA + $3\text{ }\mu\text{M}$ -U46619, $63\% \pm 4\%$ (Fig. 3), while with maximal concentrations of thrombin inducing 70–80% [^{14}C]5HT release, no effect of PMA was observed (Fig. 2).

The synergistic effects of agonist and PMA on [^{14}C]5HT secretion were not significantly affected by the addition of EGTA (5 mM) or indomethacin (Fig. 2) ($5\text{ }\mu\text{M}$), even though [Ca^{2+}]_i mobilization and [^{14}C]5HT secretion induced by agonist alone in some cases were partially or totally inhibited by addition of these agents. In EGTA-treated platelets, threshold concentrations of thrombin (< 0.03 unit/ml) induced no significant rise in [Ca^{2+}]_i or [^{14}C]5HT secretion (results not shown), although addition of PMA 10 s before thrombin resulted in a significant potentiation of [^{14}C]5HT secretion without a detectable rise in [Ca^{2+}]_i, e.g. resting [Ca^{2+}]_i, 62 ± 2 nM; PMA + 0.01 unit of thrombin/ml, 66 ± 4 nM [Ca^{2+}]_i and $39 \pm 3\%$ [^{14}C]5HT release. Similarly, in the case of U46619, the control curve for [^{14}C]5HT secretion in the presence of EGTA was shifted to the right at all concentrations of U46619 tested (results with $3\text{ }\mu\text{M}$ -U46619 alone in Fig. 3), but the [^{14}C]5HT curve obtained when PMA was added 10 s before U46619 in the presence of EGTA was superimposable with that obtained in the absence of EGTA.

Preincubation of PMA for 5 min

Longer preincubations of PMA for 5 min before agonist addition resulted in more pronounced inhibitory effects on all agonist-induced phenomena. [Ca^{2+}]_i mobilization induced by all concentrations of thrombin and U46619 tested was inhibited by 80–100% (Fig. 1) and concomitant with this, thrombin-induced release of

[^{14}C]arachidonate, TxB_2 and βTG were inhibited by 50–100% (Table 1). The level of [^{14}C]5HT release in response to thrombin and U46619 was significantly ($P < 0.001$ – 0.01) less in platelets pretreated with PMA for 5 min than in platelets pretreated with PMA for 10 s–2 min. At all agonist concentrations, therefore, this was less than the sum of that obtained when PMA and agonist were added alone (Figs. 2 and 3). At low concentrations of thrombin (< 0.02 unit/ml) and all concentrations of U46619 tested, the percentage [^{14}C]5HT release was not significantly different ($P > 0.05$) from that obtained with PMA alone or agonist alone, whichever was higher, while at submaximal to maximal concentrations of thrombin (0.05–0.2 unit/ml), a significant ($P < 0.01$ – 0.002) reduction in the percentage [^{14}C]5HT release compared with control was observed (Fig. 2). This reduction of thrombin-induced [^{14}C]5HT release by PMA was specific for PMA, as addition of U46619 (0.3–1 μM), which induced 15–20% [^{14}C]5HT release on its own, 5 min before thrombin (0.05–0.2 unit/ml), had no similar inhibitory effect (results not shown).

Pre-incubation of 4β -phorbol, the inactive parent compound of PMA [14], (16–160 nM) for 10 s–5 min before addition of thrombin (0.02–0.2 unit/ml) had no significant effect on thrombin-induced [Ca^{2+}]_i mobilization or [^{14}C]5HT secretion (results not shown).

Effect of PMA on arachidonate-induced [^{14}C]5HT release and arachidonate conversion to TxB_2

The conversion of arachidonate (500 μM) to TxB_2 measured by radioimmunoassay (cross-reactivity of arachidonate with the antisera for TxB_2 was less than 0.001%) in PRP was reduced by pretreatment of the platelets with PMA for 10 s or 5 min (Table 2). A greater degree of inhibition was observed with the longer time of pretreatment, and this was statistically significant ($P < 0.005$) compared with the vehicle control. Interestingly, the level of [^{14}C]5HT release induced by arachidonate was not significantly ($P > 0.05$) affected by a 10 s or 5 min pretreatment with PMA, although it was virtually abolished by addition of indomethacin, both in the presence and absence of PMA (Table 2).

Table 2. Effect of PMA on arachidonate-induced [^{14}C]5HT secretion and arachidonate conversion to TxB_2

All experiments were carried out in PRP [count $(2.5\text{--}3.0) \times 10^8$ /ml]. The concentrations of arachidonate, PMA and indomethacin used were 500 μM , 400 nM and 10 μM respectively. For the arachidonate experiments, PMA or vehicle was added 10 s or 5 min before arachidonate as indicated and, for the experiments with PMA alone, PMA was added 10 s or 5 min before alcohol vehicle (0.04%), which substituted for arachidonate. Indomethacin was added 60 s before PMA or vehicle. The figures represent means $\pm$ S.E.M. of data (9–12 determinations) from three similarly performed experiments, using platelets from different donors. * $P < 0.005$ compared with vehicle plus arachidonate.

Indomethacin	Pretreatment with PMA or vehicle	Arachidonate or vehicle	[^{14}C]5HT release (%)	TxB_2 formed (pmol/ 10^8 platelets)
—	Resting	—	—	1.5 ± 1.7
—	PMA (10 s)	Vehicle	11.1 ± 1	1.1 ± 1.2
—	PMA (5 min)	Vehicle	22.4 ± 2	2.5 ± 1.8
+	PMA (5 min)	Vehicle	15.0 ± 1	—
—	Vehicle (10 s)	Arachidonate	49.0 ± 3	220 ± 26
—	PMA (10 s)	Arachidonate	55.9 ± 4	118 ± 32
—	PMA (5 min)	Arachidonate	52.3 ± 3	$44 \pm 18^*$
+	Vehicle (5 min)	Arachidonate	$6.0 \pm 1^*$	—
+	PMA (5 min)	Arachidonate	$11.5 \pm 5^*$	0.5

DISCUSSION

Several recent reports have described inhibitory effects of phorbol esters on agonist-induced cellular processes such as Ca^{2+} mobilization [11,12,14,15,25], Na^+/H^+ exchange transport systems [26], inositol phospholipid synthesis [27] and breakdown [14–17], as well as effects on enzyme/receptor-regulating proteins, such as the guanine nucleotide binding proteins involved in the regulation of adenylate cyclase [28–30]. In platelets, the inhibition of agonist-induced $[\text{Ca}^{2+}]_i$ mobilization and $\text{PtdIns}(4,5)\text{P}_2$ breakdown by phorbol esters [14–17] has led to the view that protein kinase C activation could be an important 'feed-back' regulator of these agonist-induced phenomena and, consequently, agonist-induced platelet activation, which is dependent on these processes [6–10].

Our results confirm the earlier reported [14,15] inhibition of agonist-induced rise in $[\text{Ca}^{2+}]_i$ by PMA and, in addition, demonstrate a good correlation between the inhibition of this phenomenon and the inhibition of arachidonate release, TxB_2 formation and βTG release induced by thrombin. The inhibition of arachidonate conversion of TxB_2 by PMA is interesting, as it indicates an effect of PMA on some component of the cyclo-oxygenase pathway, which remains to be elucidated. Whether arachidonate conversion via the lipxygenase pathway is similarly inhibited by PMA is not known at present.

It is unlikely that inhibition of agonist-induced 5HT secretion by 5 min pretreatment with PMA is related to the inhibition of agonist-induced $[\text{Ca}^{2+}]_i$ mobilization, as our experiments with EGTA-treated platelets demonstrate a potentiation of agonist-induced 5HT secretion by 10 s pretreatments with PMA, in the absence of a detectable rise in $[\text{Ca}^{2+}]_i$. As earlier workers have shown that agonist-induced $\text{PtdIns}(4,5)\text{P}_2$ breakdown, like 5HT secretion, is significantly inhibited only with preincubation times greater than 4 min [15], inhibition of $\text{PtdIns}(4,5)\text{P}_2$ breakdown rather than inhibition of $[\text{Ca}^{2+}]_i$ mobilization may influence the effect of PMA on agonist-induced 5HT secretion.

Previous workers [10,14–17] have postulated a relationship between protein kinase C activation and the inhibitory effects of PMA, but it is interesting that our recent studies [31] with 1-oleoyl-2-acetylgllycerol have revealed that the DAG mimic has no inhibitory effect on the thrombin-induced rise in $[\text{Ca}^{2+}]_i$ levels or 5HT secretion. Although 1-oleoyl-2-acetylgllycerol may have inhibitory effects on agonist-induced $\text{PtdIns}(4,5)\text{P}_2$ breakdown [16,32] even in our system, it seems that these effects may be too weak to inhibit platelet responses such as secretion. Thus, the physiological significance of the inhibitory effects of PMA and indeed, the role of protein kinase C activation in the termination of agonist-induced responses, at least in human platelets, need to be reconsidered.

On the other hand, it may be significant that both PMA and 1-oleoyl-2-acetylgllycerol [31] were able to potentiate agonist-induced 5HT secretion in the absence of a significant rise in $[\text{Ca}^{2+}]_i$ and endogenous thromboxane formation. Although the synergistic effects of agonist and PMA on 5HT secretion in this study are only apparent with the short preincubations with PMA (10 s–2 min), the existence of synergism even with the 5 min preincubations is implied by the finding that a

5 min preincubation with PMA totally suppressed U46619 ($3\ \mu\text{M}$)-induced rise in $[\text{Ca}^{2+}]_i$ levels, but had no noticeable effect on 5HT secretion. However, the extent of 5HT secretion in this case was lower than that seen when PMA was added 10 s before U46619, and this is probably due to inhibitory effects of PMA counteracting the synergistic effects to some extent. It is noteworthy that, unlike 5HT secretion, no synergistic potentiation of βTG secretion by agonist and PMA was observed even with the short incubations with PMA, and this suggests that different mechanisms may be involved in the release of these dense- and α -granule markers.

The effects of PMA on agonist-induced platelet responses seem complex and the mechanism(s) behind its inhibitory and potentiatory effects remains to be determined. The potentiatory effects of PMA on agonist-induced 5HT secretion are, however, interesting as they are shared by 1-oleoyl-2-acetylgllycerol and, as they occur at basal $[\text{Ca}^{2+}]_i$ levels, are unlike the synergistic effects described earlier between Ca^{2+} ionophores and PMA [6,8]. These effects suggest that synergistic interactions between exogenously added agonists and endogenously formed DAG at basal $[\text{Ca}^{2+}]_i$ levels may represent what is possibly a physiologically important pathway of platelet secretion, involving both agonist and DAG in the absence of $[\text{Ca}^{2+}]_i$ mobilization.

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