Biochem. J. (1987) 243, 667–678 (Printed in Great Britain)

Effects of activation of protein kinase C on the agonist-induced stimulation and inhibition of cyclic AMP formation in intact human platelets

Kimberley A. WILLIAMS, Wanda MURPHY and Richard J. HASLAM
Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Jakobs, Bauer & Watanabe [(1985) Eur. J. Biochem. **151**, 425–430] reported that treatment of platelets with phorbol 12-myristate 13-acetate (PMA) prevented GTP- and agonist-induced inhibition of adenylate cyclase in membranes from the platelets. This was attributed to the phosphorylation of the inhibitory guanine nucleotide-binding protein (G_i) by protein kinase C. In the present study, the effects of PMA on cyclic [^{3}H]AMP formation and protein phosphorylation were studied in intact human platelets labelled with [^{3}H]adenine and [^{32}P]P_i. Incubation mixtures contained indomethacin to block prostaglandin synthesis, phosphocreatine and creatine kinase to remove ADP released from the platelets, and 3-isobutyl-1-methylxanthine to inhibit cyclic AMP phosphodiesterases. Under these conditions, PMA partially inhibited the initial formation of cyclic [^{3}H]AMP induced by prostaglandin E_1 (PGE_1), but later enhanced cyclic [^{3}H]AMP accumulation by blocking the slow decrease in activation of adenylate cyclase that follows addition of PGE_1. PMA had more marked and exclusively inhibitory effects on cyclic [^{3}H]AMP formation induced by prostaglandin D_2 and also inhibited the action of forskolin. Adrenaline, high thrombin concentrations and, in the absence of phosphocreatine and creatine kinase, ADP inhibited cyclic [^{3}H]AMP formation induced by PGE_1. The actions of adrenaline and thrombin were attenuated by PMA, but that of ADP was little affected, suggesting differences in the mechanisms by which these agonists inhibit adenylate cyclase. *sn*-1,2-Dioctanoylglycerol (diC_8) had effects similar to those of PMA. The actions of increasing concentrations of PMA or diC_8 on the modulation of cyclic [^{3}H]AMP formation by PGE_1 or adrenaline correlated with intracellular protein kinase C activity, as determined by ^{32}P incorporation into the 47 kDa substrate of the enzyme. Parallel increases in phosphorylation of 20 kDa and 39–41 kDa proteins were also observed. Platelet-activating factor, [Arg^8]vasopressin and low thrombin concentrations, all of which inhibit adenylate cyclase in isolated platelet membranes, did not affect cyclic [^{3}H]AMP formation in intact platelets. However, the activation of protein kinase C by these agonists was insufficient to account for their failure to inhibit cyclic [^{3}H]AMP formation. Moreover, high thrombin concentrations simultaneously activated protein kinase C and inhibited cyclic [^{3}H]AMP formation. The results show that, in the intact platelet, the predominant effects of activation of protein kinase C on adenylate cyclase activity are inhibitory, suggesting actions additional to inactivation of G_i.

INTRODUCTION

Aggregation and degranulation of platelets by physiological stimuli are mediated by specific receptors that generate one or more of several intracellular signals. In this regard, the agonist-induced hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate by phospholipase C, to yield diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate, which releases Ca^{2+} ions from the endoplasmic reticulum into the cytosol, is of particular importance [1,2]. In the platelet, diacylglycerol and Ca^{2+} ions can act either independently or synergistically to promote platelet aggregation and degranulation [1,3]. Agonists that have been shown to stimulate phosphoinositide hydrolysis include thrombin, PAF, AVP and stable analogues of prostaglandin endoperoxides [4,5]. Other agonists, notably ADP [5,6] and adrenaline [5,7], do not directly activate phospholipase C. ADP does, however, stimulate the entry of extracellular Ca^{2+} ions [8], and both ADP and adrenaline may enhance Na^+/H^+ exchange [9]. In addition, both ADP and adrenaline can inhibit adenylate cyclase in intact platelets [10] and in platelet membrane preparations [11,12], though this effect plays no role in mediating platelet responses and serves only to restrict the inhibition of platelet function by activators of adenylate cyclase, such as PGE_1 [10,13]. Although AVP [14], PAF [15] and prostaglandin endoperoxide analogues [16] inhibit the adenylate cyclase activity of isolated platelet membranes, they do not affect cyclic AMP formation in intact human platelets under conditions in which effects of released ADP are excluded [14,17,18]. In discussion of these phenomena [17], we have noted an apparent correlation between the ability of agonists to activate phospholipase C and their relative failure to inhibit adenylate cyclase in intact human platelets.

Abbreviations used: PAF, synthetic platelet-activating factor, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; AVP, [Arg^8]vasopressin; PGE_1, prostaglandin E_1; PGD_2, prostaglandin D_2; IBMX, 3-isobutyl-1-methylxanthine; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; diC_8, *sn*-1,2-dioctanoylglycerol; DMSO, dimethyl sulphoxide.

However, thrombin, which inhibits the adenylate cyclase activity of platelet membranes [19], has been reported to decrease cyclic AMP formation in intact platelets [20].

Activation of protein kinase C may not only promote cell responses, but may also exert negative-feedback effects on signal transduction [21,22]. The tumour promoter, PMA, which stimulates protein kinase C by substituting for diacylglycerol [23], has been particularly valuable in studies of these effects. Thus preincubation with PMA has been found to block the activation of phospholipase C in a wide variety of cells, including platelets [24–26]. PMA has also been shown either to inhibit [27–31] or to enhance [31–34] hormone-activated adenylate cyclase, the precise effect depending on the cell and agonist studied. Decreases in cyclic AMP formation in the presence of PMA have been observed in intact platelets [35,36], but were attributed to the release of ADP [36]. In contrast, Jakobs *et al.* [34], using membranes from control and PMA-treated platelets, found that, in the presence of GTP, PMA enhanced the activation of adenylate cyclase by PGE_1 and diminished inhibition of the enzyme by adrenaline. Both effects were attributed to impairment of signal transduction by the inhibitory guanine-nucleotide-binding protein, G_i, the α-subunit of which was phosphorylated by protein kinase C [37]. Jakobs *et al.* [34] suggested that this action of protein kinase C may explain why agonists that activate phospholipase C may not inhibit adenylate cyclase in intact platelets. In the present work, we sought to validate this hypothesis by studying the relationship between activation of protein kinase C and cyclic AMP formation in intact human platelets. However, the results differed substantially from those predicted from experiments with isolated membrane preparations [34]. Some of our findings have been published in a preliminary form [38].

EXPERIMENTAL

Materials

[2,8-^{3}H]Adenine (40 Ci/mmol) was from ICN Radiochemicals (Irvine, CA, U.S.A.). Cyclic [8-^{14}C]AMP (50 mCi/mmol) and carrier-free [^{32}P]P_i were from New England Nuclear Canada Ltd. (Lachine, Que., Canada). ADP, (−)-adrenaline (+)-bitartrate, AVP, indomethacin, phosphocreatine (disodium salt), creatine kinase, IBMX, PMA, 4α-PDD, phorbol, heparin, Hepes, bovine serum albumin (fraction V) and neutral alumina (WN3) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Forskolin was from Behring Diagnostics (San Diego, CA, U.S.A.) and diC_8 from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Dowex 50 resin (AG 50W-X8, 200–400 mesh, H^+ form) was from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ont., Canada), and Ready-Solv HP/b scintillant was from Beckman Instruments (Toronto, Ont., Canada). Materials used in SDS/polyacrylamide-gel electrophoresis were from sources listed elsewhere [39]. Potato apyrase was prepared as described in [40]. The following were generously given: PGE_1 and PGD_2 by Dr. J. Pike of the Upjohn Co. (Kalamazoo, MI, U.S.A.), synthetic PAF(1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) by Dr. H. R. Baumgartner of F. Hoffmann–La Roche and Co. (Basel, Switzerland), human α-thrombin (2700 units/mg) by Dr. J. W. Fenton II of New York State Department of Health (Albany, NY, U.S.A.), and (−)-propranolol by I.C.I. (Macclesfield, U.K.).

Preparation of washed human platelets labelled with [^{3}H]adenine alone or with both [^{3}H]adenine and [^{32}P]P_i

Washed platelets were prepared by a modification of the method of Mustard *et al.* [41]. Venous blood was collected into 37 ml of ACD anticoagulant [42] to give a final volume of 250 ml. The citrated blood was centrifuged at 160 $g_{av.}$ for 15 min at room temperature to give platelet-rich plasma. This was repeated and the pooled material was centrifuged at 1300 $g_{av.}$ for 15 min. After removal of residual red and white cells, the platelets in the pellet were resuspended in 40 ml of Tyrode's solution supplemented with 5 mM-Hepes (adjusted to pH 7.35 with NaOH), 0.35% bovine serum albumin, 60 μg of apyrase/ml and 50 units of heparin/ml and incubated for 20 min at 37 °C. This suspension was centrifuged at 1100 $g_{av.}$ for 10 min at 37 °C, and the pellet (2×10^{10}–4×10^{10} platelets) was resuspended in 20 ml of the same medium without heparin but containing 2 μM-[^{3}H]adenine (10 Ci/mmol). After incubation for 90 min at 37 °C, the platelets were then centrifuged as before and finally resuspended at 3×10^8–5×10^8/ml in Tyrode's solution containing 5 mM-Hepes (pH 7.35), 0.35% bovine serum albumin and only 6 μg of apyrase/ml. The last prevents stimulation of the platelets by traces of ADP that may be released during storage of the suspension, but does not block the immediate effects of addition of higher (micromolar) concentrations of ADP.

Platelets were labelled with both [^{3}H]adenine and [^{32}P]P_i by a modification of the above method. For incubation with 2 μM-[^{3}H]adenine, the platelets were suspended in 10 ml of phosphate-free Tyrode's solution supplemented with Hepes buffer, bovine serum albumin and apyrase as before, and were incubated for 30 min at 37 °C. After centrifugation of the platelets and their resuspension in 8 ml of the same phosphate-free medium, they were incubated for 60 min at 37 °C with 2 mCi of carrier-free [^{32}P]P_i. The platelets were then centrifuged, washed once more in medium containing phosphate and finally resuspended at 3×10^8–5×10^8/ml in albumin-free Tyrode's solution containing phosphate, Hepes buffer and 6 μg of apyrase/ml.

Incubation of labelled platelets

Experiments were carried out about 30 min after final resuspension of the platelets. Samples of platelet suspension (409 μl) containing 1.2×10^8–2.0×10^8 platelets were mixed with 10 μl of 0.154 M-NaCl containing 500 μM-indomethacin, 10 μl of 100 mM-phosphocreatine and 10 μl of 0.154 M-NaCl containing 5000 units of creatine kinase/ml, and were incubated for 5 min at 37 °C. Then 1 μl of DMSO or of phorbol ester or diC_8 dissolved in DMSO was added to each experimental sample. After a further period of incubation at 37 °C (usually 1 min), 20 μl of 0.154 M-NaCl containing any activators and/or inhibitors of adenylate cyclase was added with 40 μl of 0.154 M-NaCl containing 12.5 mM-IBMX. Incubations were stopped, usually 0.5 min later, by addition of 1 ml of 30% or 15% (w/v) trichloroacetic acid. The lower concentration was used when platelet protein was analysed by SDS/polyacrylamide-gel electrophoresis. The total incubation volume before addition of

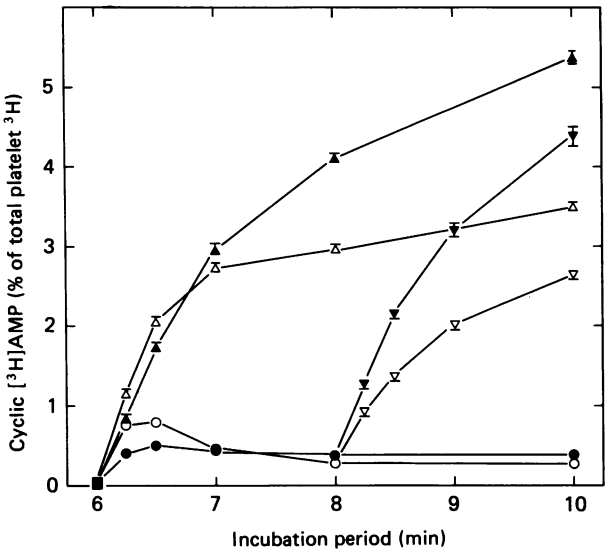

Fig. 1. Effects of PMA on the increases in platelet cyclic [^{3}H]AMP caused by PGE_1 in the absence and presence of IBMX

Platelets labelled with [^{3}H]adenine were first incubated for 5 min with indomethacin, phosphocreatine and creatine kinase and then for 1 min with DMSO (open symbols) or 1 μM-PMA (solid symbols) (see the Experimental section). Basal cyclic [^{3}H]AMP at this time is shown (□, ■). Cyclic [^{3}H]AMP formation was then initiated by addition of either 0.2 μM-PGE_1 (○, ●) or 0.2 μM-PGE_1 and 1 mM-IBMX (△, ▲). Incubations were terminated after a further 0.25, 0.5, 1, 2 or 4 min. In some samples, 1 mM-IBMX was added 2 min after 0.2 μM-PGE_1 and incubation was continued for 0.25, 0.5, 1 or 2 min (▽, ▼). All incubations were performed in triplicate; values are means ± S.E.M.

trichloroacetic acid was 500 μl and, unless stated otherwise, contained 10 μM-indomethacin, 2 mM-phosphocreatine, 100 units of creatine kinase/ml and 0.2% DMSO, as well as any other additions. In a few experiments, incubation mixtures were stirred in a Payton Aggregometer (Scarborough, Ont., Canada) to record platelet shape change and aggregation.

Isolation and measurement of platelet cyclic [^{3}H]AMP

After addition of trichloroacetic acid, cyclic [^{14}C]AMP (1000 or 2000 d.p.m.) was added to all the samples to monitor the recovery of cyclic [^{3}H]AMP. Labelled cyclic AMP in the supernatant from each sample was isolated by chromatography on alumina and Dowex 50 resin [43]. The final eluates were freeze-dried and counted for ^{3}H, ^{14}C and, when present, ^{32}P in mixtures containing 0.5 ml of water and 8 ml of HP/b scintillant. Results were corrected for background radioactivity and for channel cross-over of ^{14}C and ^{32}P. The counting efficiency of ^{3}H was 17% and that of ^{14}C was 52%. After correction for recovery of cyclic [^{14}C]AMP, the cyclic [^{3}H]AMP found was expressed as a percentage of the total ^{3}H in the platelets present in the experimental samples. This was determined by counting the ^{3}H in samples of platelet suspension and of suspending medium obtained by centrifugation. About 95% of the ^{3}H in platelet suspensions was intracellular. Unexpectedly, cyclic AMP isolated from ^{32}P-labelled platelets contained ^{32}P, implying significant labelling of platelet ATP in the α-position. Cyclic [^{32}P]AMP was proportional to cyclic [^{3}H]AMP, but only the latter was used in this study.

Measurement of protein phosphorylation in intact platelets

The protein precipitated by trichloroacetic acid from experimental samples containing ^{32}P-labelled platelets was dissolved in electrophoresis sample buffer [44]. After neutralization of residual acid, the samples were heated at 100 °C for 3 min. Equal amounts of protein from the experimental samples (about 50 μg) were analysed by SDS/polyacrylamide-slab-gel electrophoresis, with 13% (w/v) acrylamide in the separating gel [39]. Labelled polypeptides were located by autoradiography, and selected polypeptides were cut from the dried gels and counted for Čerenkov radiation [45]. The M_r values assigned to ^{32}P-labelled polypeptides are either those determined previously [45] or were determined from the latter by interpolation.

Statistics

Incubations were performed in triplicate in each experiment. Tables and Figures are derived from single experiments and give mean values ± S.E. of the mean, difference or quotient, as appropriate. The effects described were obtained in at least three separate experiments, and statistical information indicating the variation encountered is given in the text. Qualitatively similar changes in cyclic [^{3}H]AMP formation were observed in singly or doubly labelled platelet preparations. However, the statistics presented are derived from only one or the other type of preparation, as indicated. The significance of changes was evaluated by two-sided unpaired or paired t tests.

RESULTS

Effects of PMA and analogues on the agonist-induced stimulation of cyclic [^{3}H]AMP formation

Experiments were carried out in the presence of indomethacin, to prevent the formation of prostaglandins or thromboxane A_2, and of phosphocreatine and creatine kinase, to remove any ADP released from the platelets. These precautions were necessary to ensure that changes in platelet cyclic [^{3}H]AMP formation were due solely to direct effects of the added compounds. Under these conditions, treatment of platelets labelled with [^{3}H]adenine alone with 1 μM-PMA for 1 min decreased their basal content of cyclic [^{3}H]AMP (0.038 ± 0.002% of platelet ^{3}H) by 18 ± 1% (mean values ± S.E.M., eight expts.). The initial rate of accumulation of cyclic [^{3}H]AMP induced by addition of both PGE_1 and IBMX was also diminished (Fig. 1). Thus PMA inhibited the accumulation of cyclic [^{3}H]AMP observed after 15 s by 24 ± 3% (mean ± S.E.M., five expts.) and that observed after 30 s by 16 ± 3% (mean ± S.E.M., eight expts.). Cyclic [^{3}H]AMP accumulation in the presence of IBMX was linear for about 30 s and reflects the endogenous adenylate cyclase activity of the intact platelet [43]. At later times, cyclic [^{3}H]AMP increased more slowly and, after 1 min, the inhibitory effect of PMA was replaced by stimulation (Fig. 1). In the absence of IBMX, PGE_1 increased platelet cyclic [^{3}H]AMP to a maximum after about 30 s, and the amount then declined to about 40% of this value after 2 min. PMA inhibited both the initial

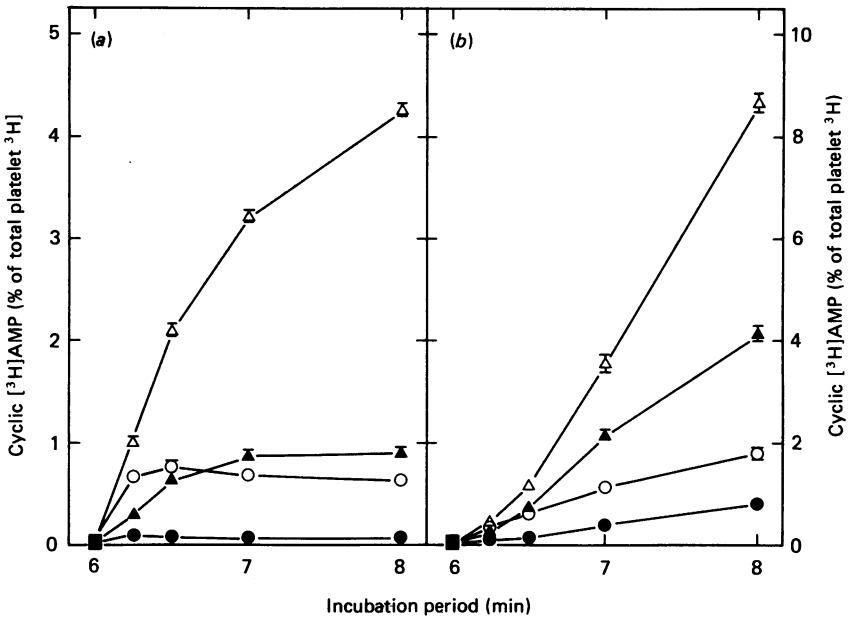

Fig. 2. Effects of PMA on the increases in platelet cyclic [^{3}H]AMP caused by PGD_2 and by forskolin in the absence and presence of IBMX

Samples of a suspension of platelets labelled with [^{3}H]adenine were first incubated for 5 min with indomethacin, phosphocreatine and creatine kinase and then for 1 min with DMSO (open symbols) or 1 μM-PMA (closed symbols) (see the Experimental section). Basal cyclic [^{3}H]AMP at this time is shown (□, ■). Cyclic [^{3}H]AMP formation was then initiated by the following additions: (*a*) 0.2 μM-PGD_2 (○, ●) or 0.2 μM-PGD_2 and 1 mM-IBMX (△, ▲); (*b*) 20 μM-forskolin (○, ●) or 20 μM-forskolin and 1 mM-IBMX (△, ▲). Incubations were terminated after 0.25, 0.5, 1 or 2 min. All incubations were performed in triplicate; values are means ± S.E.M.

Table 1. Effects of PMA on the formation of cyclic [^{3}H]AMP induced by PGE_1 and on the inhibition of this reaction by adrenaline and by ADP

Samples of a suspension of platelets labelled with [^{3}H]adenine were incubated for 5 min with indomethacin in the absence or presence of phosphocreatine (PC) and creatine kinase (CK) (see the Experimental section) and then for a further 1 min with DMSO, 1 μM-PMA, 1 μM-phorbol or 1 μM-4α-PDD, as indicated. Cyclic [^{3}H]AMP formation was then initiated by addition of 0.2 μM-PGE_1 and 1 mM-IBMX with or without 5 μM-adrenaline or 10 μM-ADP. Propranolol (10 μM) was included with adrenaline. The incubations were terminated after 0.5 min by addition of trichloroacetic acid. All incubations were performed in triplicate. The basal value for platelet cyclic [^{3}H]AMP was subtracted in calculation of increases (means ± S.E. of the difference). Percentage inhibitions of cyclic [^{3}H]AMP formation by adrenaline or ADP are given (means ± S.E. of the quotient).

Initial additions		Additions with PGE_1 and IBMX	Increase in cyclic [^{3}H]AMP (% of total platelet ^{3}H)	Inhibition of cyclic [^{3}H]AMP formation (%)
0 min	5 min	6 min		
Indomethacin	DMSO	None	1.266 ± 0.022	–
		Adrenaline	0.196 ± 0.011	84 ± 1
		ADP	0.301 ± 0.011	76 ± 1
	PMA	None	0.718 ± 0.062	–
		Adrenaline	0.465 ± 0.014	35 ± 6
		ADP	0.235 ± 0.014	67 ± 3
Indomethacin + PC + CK	DMSO	None	1.333 ± 0.013	–
		Adrenaline	0.190 ± 0.002	86 ± 1
		ADP	1.162 ± 0.018	13 ± 2
	PMA	None	0.899 ± 0.032	–
		Adrenaline	0.586 ± 0.017	35 ± 3
	Phorbol	None	1.302 ± 0.060	–
		Adrenaline	0.188 ± 0.002	86 ± 1
	4α-PDD	None	1.282 ± 0.049	–
		Adrenaline	0.177 ± 0.004	86 ± 1

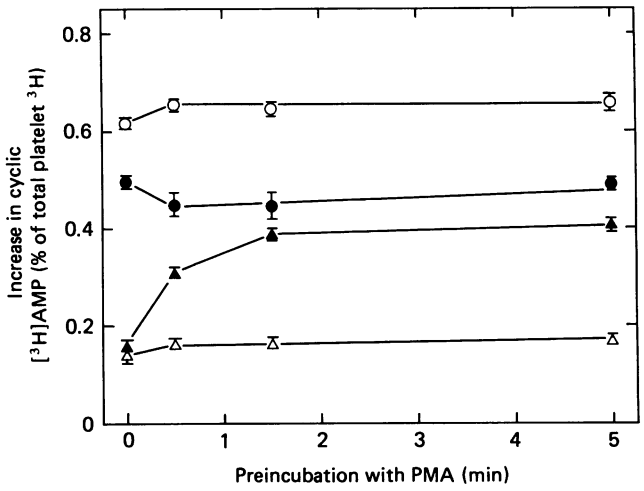

Fig. 3. Effect of the period of preincubation with PMA on the increases in platelet cyclic [^{3}H]AMP caused by PGE_1 added without or with adrenaline

Samples of a suspension of platelets labelled with [^{3}H]adenine were first incubated for 5 min with indomethacin, phosphocreatine and creatine kinase (see the Experimental section). After addition of DMSO (open symbols) or 100 nM-PMA (closed symbols), the samples were incubated for a further 0, 0.5, 1.5 or 5 min before cyclic [^{3}H]AMP formation was initiated by addition of 0.2 μM-PGE_1 with 1 mM-IBMX (○, ●) or of 0.2 μM-PGE_1, 5 μM-adrenaline and 10 μM-propranolol with 1 mM-IBMX (△, ▲). Incubations were stopped after 0.5 min by addition of trichloroacetic acid. All were performed in triplicate. The basal values for cyclic [^{3}H]AMP found in the absence of IBMX, PGE_1 or adrenaline at each time point are subtracted from all values shown (means ± S.E. of the difference).

increase in cyclic [^{3}H]AMP and the spontaneous decrease, so that after 1 min the amount of cyclic [^{3}H]AMP was again higher in the presence of PMA than in its absence (Fig. 1). Measurement of initial rates of cyclic [^{3}H]AMP accumulation in the presence of IBMX indicated that platelet adenylate cyclase activity decreased markedly during the 2 min after exposure of control platelets to PGE_1, but did not change from the initial inhibited value in platelets that had been preincubated with 1 μM-PMA (Fig. 1). Thus PMA had two actions on the activation of platelet adenylate cyclase of PGE_1, namely an inhibitory effect detectable immediately after addition of PGE_1, and an apparent stimulatory effect attributable to suppression of the normal decrease in the response to PGE_1. Similar experiments on the activation of adenylate cyclase in intact platelets by PGD_2, which showed little decrease in incubations of up to 2 min, demonstrated that PMA exerted potent purely inhibitory effects on cyclic [^{3}H]AMP accumulation (Fig. 2*a*). With forskolin, which showed a different, more progressive, pattern of cyclic [^{3}H]AMP accumulation, PMA caused a moderate inhibition at all incubation times (Fig. 2*b*).

Whereas addition of both phosphocreatine and creatine kinase almost abolished the inhibition of PGE_1-induced cyclic [^{3}H]AMP formation by 10 μM-ADP, these additions had only a small effect on the inhibitory action of PMA, which therefore cannot be attributed to the release of platelet ADP (Table 1). In further support of this view, the inhibitory action of PMA was observed when the compound was added with PGE_1 and was optimal when added only 30 s before PGE_1 (Fig. 3). Inhibition of cyclic [^{3}H]AMP formation by PMA was structurally specific and was not seen with phorbol or 4α-PDD (Table 1). DiC_8, on the other hand, which reproduces many of the effects of PMA on platelets [46], inhibited cyclic [^{3}H]AMP formation induced by addition of PGE_1 and IBMX (Fig. 4). Thus, in doubly labelled platelet preparations, 40 μM-diC_8 inhibited cyclic [^{3}H]AMP formation by 28±9% (mean±S.E.M., four expts.), whereas 100 nM-PMA inhibited by 25±4%, mean±S.E.M., eight expts.). In contrast, low diC_8 concentrations caused a weak but significant stimulation of cyclic [^{3}H]AMP formation (19±6% at 1 μM, four expts.; $P < 0.05$, paired t test) that was rarely seen with PMA.

Effects of PMA and analogues on the agonist-induced inhibition of cyclic [^{3}H]AMP formation

Preincubation of platelets with 10 nM–1 μM-PMA diminished the α-adrenergic inhibition by adrenaline of the cyclic [^{3}H]AMP accumulation caused by addition of PGE_1 with IBMX (Table 1, Figs. 3 and 4). This was only partly due to inhibition of the action of PGE_1 by PMA, as cyclic [^{3}H]AMP formation was considerably greater in the presence of PMA, when adrenaline was added. The latter effect of PMA, which was optimal after about 1.5 min, developed more slowly than its action on PGE_1-induced cyclic [^{3}H]AMP formation (Fig. 3). In four experiments with singly labelled platelets, the inhibition of PGE_1-induced cyclic [^{3}H]AMP formation by 5 μM-adrenaline decreased from 79±3% to 22±5% (mean values±S.E.M.) after preincubation of the platelets with 1 μM-PMA for 1 min. Both the inhibitory action of adrenaline and the suppression of its effects by PMA were unaffected by phosphocreatine and creatine kinase, indicating that release of platelet ADP played no role in these phenomena (Table 1). Neither phorbol nor 4α-PDD added at 1 μM had any effect on the inhibition of cyclic [^{3}H]AMP formation by adrenaline (Table 1), but preincubation of the platelets with 4–40 μM-diC_8 had effects similar to those of 1–100 nM-PMA (Fig. 4). Higher diC_8 concentrations had non-specific inhibitory effects on cyclic [^{3}H]AMP formation (Fig. 4). Adrenaline also inhibited cyclic [^{3}H]AMP accumulation in platelets caused by addition of forskolin with IBMX; this effect, too, was attenuated by PMA (Table 2).

In the absence of phosphocreatine and creatine kinase, addition of 10 μM-ADP was almost as effective as 5 μM-adrenaline in inhibiting PGE_1-induced cyclic [^{3}H]AMP formation (Table 1). However, preincubation of the platelets with 1 μM-PMA for 1 min had only a small effect on this action of ADP, decreasing the inhibition from 72±4% to 59±6% (mean values±S.E.M.) in three experiments identical with that shown in Table 1. Although this effect was statistically significant ($P < 0.05$, paired t test), PMA appeared to act mainly by inhibiting the action of PGE_1, rather than that of ADP.

High concentrations of thrombin (2–5 units/ml) decreased the PGE_1-induced accumulation of cyclic [^{3}H]AMP in platelets incubated with indomethacin, phosphocreatine and creatine kinase, indicating that thrombin itself inhibited platelet adenylate cyclase activity (Table 2). This effect was also attenuated by preincubation of the platelets with PMA, though not as

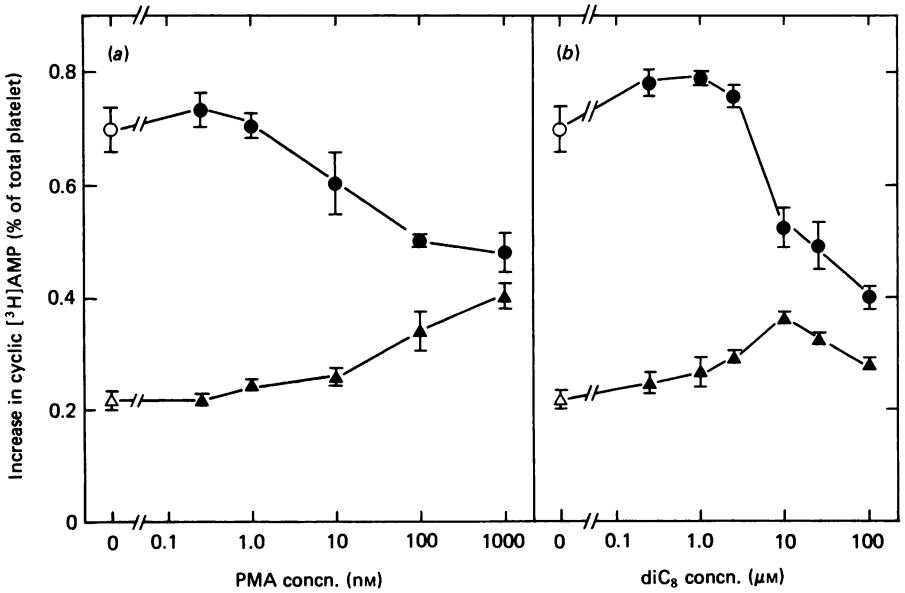

Fig. 4. Effects of different concentrations of PMA (*a*) and of diC_8 (*b*) on the increases in platelet cyclic [^{3}H]AMP caused by PGE_1 added without or with adrenaline

Washed platelets were labelled by incubation with both [^{3}H]adenine and [^{32}P]P_i, as described in the Experimental section. Samples were incubated for 5 min with indomethacin, phosphocreatine and creatine kinase, and then for 1 min with DMSO (open symbols) or the indicated concentrations of PMA or diC_8 (solid symbols). Cyclic [^{3}H]AMP formation was then initiated by addition of 0.2 μM-PGE_1 with 1 mM-IBMX (○, ●) or of 0.2 μM-PGE_1, 5 μM-adrenaline and 10 μM-propranolol with 1 mM-IBMX (△, ▲). Incubations were stopped after 0.5 min by addition of trichloroacetic acid. All were carried out in triplicate. The basal value for cyclic [^{3}H]AMP was subtracted in calculation of the increases shown (means ± S.E. of the difference).

effectively as was the action of adrenaline. Thus preincubation of doubly labelled platelets with 100 nM-PMA for 1 min decreased the inhibition of cyclic [^{3}H]AMP formation by 2 units of thrombin/ml from 64 ± 5% to 40 ± 6% (mean values ± S.E.M., three expts.), whereas under the same conditions the inhibitory effect of adrenaline decreased from 67 ± 3% to 29 ± 4% (mean values ± S.E.M., seven expts.). In contrast, lower concentrations of thrombin (0.2 unit/ml) and high concentrations of PAF (1 μM) or AVP (0.4 μM) had no effect on the accumulation of cyclic [^{3}H]AMP caused by addition of PGE_1 and IBMX, in either the absence or the presence of PMA (Table 2). Similarly, AVP did not affect cyclic [^{3}H]AMP formation induced by forskolin. These negative results were obtained despite the fact that the same platelets responded to low thrombin, PAF or AVP with a change in shape and some aggregation, when stirred in incubation mixtures containing indomethacin, phosphocreatine and creatine kinase, but no PGE_1 or IBMX.

Relationships between the effects of PMA, diC_8 and physiological stimuli on protein kinase C and their effects on cyclic [^{3}H]AMP formation

Treatment of ^{32}P-labelled platelets with either PMA or diC_8 caused major increases in ^{32}P incorporation into protein P47 (the principal substrate of protein kinase C in platelets [1,39]), into myosin light-chain isoforms (P20a,b) and into polypeptides of 39–41 kDa designated P39–41 (Fig. 5). The last normally migrated as a doublet, but were compressed into a single band in gels from incubation mixtures containing creatine kinase (Fig. 5). These phosphorylation reactions were detected with 10 nM-PMA or 4 μM-diC_8 and were maximal with 1 μM-PMA or 100 μM-diC_8. With the higher concentrations of PMA or diC_8, increased labelling of many minor polypeptides was also detected (Fig. 5). Phorbol had none of these effects. Addition of PGE_1 and IBMX 1 min after PMA or diC_8 did not modify the above phosphorylation reactions during the additional 30 s required for measurement of cyclic [^{3}H]AMP formation (Fig. 5, Table 2). Incorporation of ^{32}P into protein P47 was utilized as the most sensitive means of quantifying the endogenous activity of protein kinase C in the platelets. Within the error of measurement, the labelling of the other substrates noted above was proportional to that of P47.

The relationships between the activation of protein kinase C and changes in cyclic [^{3}H]AMP formation were investigated in platelets labelled with both [^{3}H]adenine and [^{32}P]P_i. On addition of increasing concentrations of PMA, a reciprocal relationship was observed between the incorporation of ^{32}P into protein P47 and the inhibition by adrenaline of PGE_1-induced cyclic [^{3}H]AMP formation (Fig. 6*a*). As described above, the latter effect consisted of two components (see Fig. 4); both showed the same relationship to the phosphorylation of protein P47. Similar results were obtained in platelets treated with 4–40 μM-diC_8 (Fig. 6*b*). These findings are consistent with the view that the major effects of both PMA and diC_8 on platelet adenylate cyclase are mediated by protein kinase C.

To determine whether activation of protein kinase C by physiological agonists was sufficient to affect cyclic [^{3}H]AMP formation, both were studied in the same platelets and were compared with the actions of PMA

Table 2. Effects of different aggregating agents on the increases in platelet cyclic [^{3}H]AMP caused by PGE_1 or by forskolin in the absence and presence of PMA

Washed platelets were labelled by incubation with both [^{3}H]adenine and [^{32}P]P_i. Samples were first incubated for 5 min with indomethacin, phosphocreatine and creatine kinase, followed by 1 min with DMSO or 100 nM-PMA (see the Experimental section). Cyclic [^{3}H]AMP formation was then initiated by addition of 0.2 μM-PGE_1 with 1 mM-IBMX or of 20 μM-forskolin with 1 mM-IBMX at the same time as the aggregating agents indicated. Propranolol (10 μM) was included with adrenaline. After 0.5 min, the incubations were stopped with trichloroacetic acid and cyclic [^{3}H]AMP was determined. All incubations were performed in triplicate. The basal value for cyclic [^{3}H]AMP was subtracted in calculation of the increases shown (means ± S.E. of the difference). The percentage inhibitions of cyclic [^{3}H]AMP formation by the aggregating agents are given (means ± S.E. of the quotient). Significant effects are indicated (*$P < 0.05$; **, $P < 0.01$, unpaired t test).

Additions		Increase in cyclic [^{3}H]AMP (% of total platelet ^{3}H)	Inhibition of cyclic [^{3}H]AMP formation (%)
5 min	6 min		
DMSO	PGE_1 + IBMX	0.729 ± 0.020	–
	PGE_1 + IBMX + PAF (1 μM)	0.763 ± 0.017	−4 ± 4
	PGE_1 + IBMX + AVP (0.4 μM)	0.734 ± 0.022	−1 ± 4
	PGE_1 + IBMX + thrombin (0.2 unit/ml)	0.659 ± 0.022	10 ± 4
	PGE_1 + IBMX + thrombin (2 units/ml)	0.241 ± 0.017	67 ± 2**
	PGE_1 + IBMX + thrombin (5 units/ml)	0.191 ± 0.003	74 ± 1**
	PGE_1 + IBMX + adrenaline (5 μM)	0.289 ± 0.023	60 ± 3**
	Forskolin + IBMX	0.840 ± 0.009	–
	Forskolin + IBMX + AVP (0.4 μM)	0.823 ± 0.032	2 ± 4
	Forskolin + IBMX + adrenaline (5 μM)	0.417 ± 0.007	50 ± 1**
PMA	PGE_1 + IBMX	0.585 ± 0.027	–
	PGE_1 + IBMX + PAF (1 μM)	0.662 ± 0.011	−13 ± 6
	PGE_1 + IBMX + AVP (0.4 μM)	0.565 ± 0.035	3 ± 7
	PGE_1 + IBMX + thrombin (0.2 unit/ml)	0.614 ± 0.015	−5 ± 5
	PGE_1 + IBMX + thrombin (2 units/ml)	0.337 ± 0.027	42 ± 5**
	PGE_1 + IBMX + thrombin (5 units/ml)	0.351 ± 0.021	40 ± 4**
	PGE_1 + IBMX + adrenaline (5 μM)	0.465 ± 0.022	21 ± 5*
	Forskolin + IBMX	0.614 ± 0.034	–
	Forskolin + IBMX + AVP (0.4 μM)	0.530 ± 0.021	14 ± 6
	Forskolin + IBMX + adrenaline (5 μM)	0.458 ± 0.014	25 ± 5*

(Tables 2 and 3). In the absence of PGE_1 and IBMX, optimal concentrations of PAF or AVP or a low concentration of thrombin (0.2 unit/ml) caused significant increases in the phosphorylation of protein P47 and of the myosin light-chain isoforms (P20a,b), but these effects were much smaller than observed with high thrombin concentrations (2–5 units/ml), which also induced detectable increases in the phosphorylation of P39–41 (Fig. 7, Table 3). Adrenaline, on the other hand, did not affect protein phosphorylation in this system. At 100 nM, PMA had effects similar to those of high thrombin concentrations on the phosphorylation of P47 and P39–41, but was less effective with P20a,b (Fig. 7), presumably because thrombin stimulates phosphorylation of the latter by both myosin light-chain kinase and protein kinase C [47]. PMA (100 nM) and thrombin (2 units/ml) did not have additive effects on the phosphorylation of P47 (results not shown). Of particular interest was the observation that 10 nM-PMA, which had only relatively small effects on cyclic [^{3}H]AMP formation in the presence or absence of adrenaline (Fig. 4*a*), caused an appreciably greater phosphorylation of P47 than did PAF, AVP or low thrombin (Fig. 7). This suggests that the latter agonists would be unlikely to modify platelet adenylate cyclase activity by activation of protein kinase C, even in the absence of PGE_1 and IBMX. When PGE_1 and IBMX were present, as was essential for measurement of changes in cyclic [^{3}H]AMP formation, no significant phosphorylation of protein P47 was detected after addition of PAF, AVP or low thrombin, and that caused by high thrombin concentrations was partly inhibited (Table 3). A similar experiment in which platelets were incubated with these agonists for 6 s or 12 s revealed no evidence for even a transient activation of protein kinase C. Forskolin and IBMX also blocked the phosphorylation of P47 induced by AVP (Table 3). Thus, the failure of PAF, AVP and low thrombin to inhibit cyclic [^{3}H]AMP formation in intact platelets cannot be accounted for by an activation of protein kinase C.

DISCUSSION

The predominant effects of PMA on cyclic [^{3}H]AMP formation in intact platelets were inhibitory, though the extent of inhibition varied with the activator of adenylate cyclase used. As the initial linear rates of cyclic [^{3}H]AMP accumulation immediately after addition of IBMX were measured, these effects must reflect an inhibition of adenylate cyclase rather than an activation of cyclic AMP phosphodiesterase. Inhibition by PMA of cyclic AMP formation induced by PGE_1 [35] or by prostacyclin or forskolin [36] has been observed previously, but in the latter study was largely prevented by apyrase, suggesting that it was mediated by the secretion of ADP from the platelet dense granules.

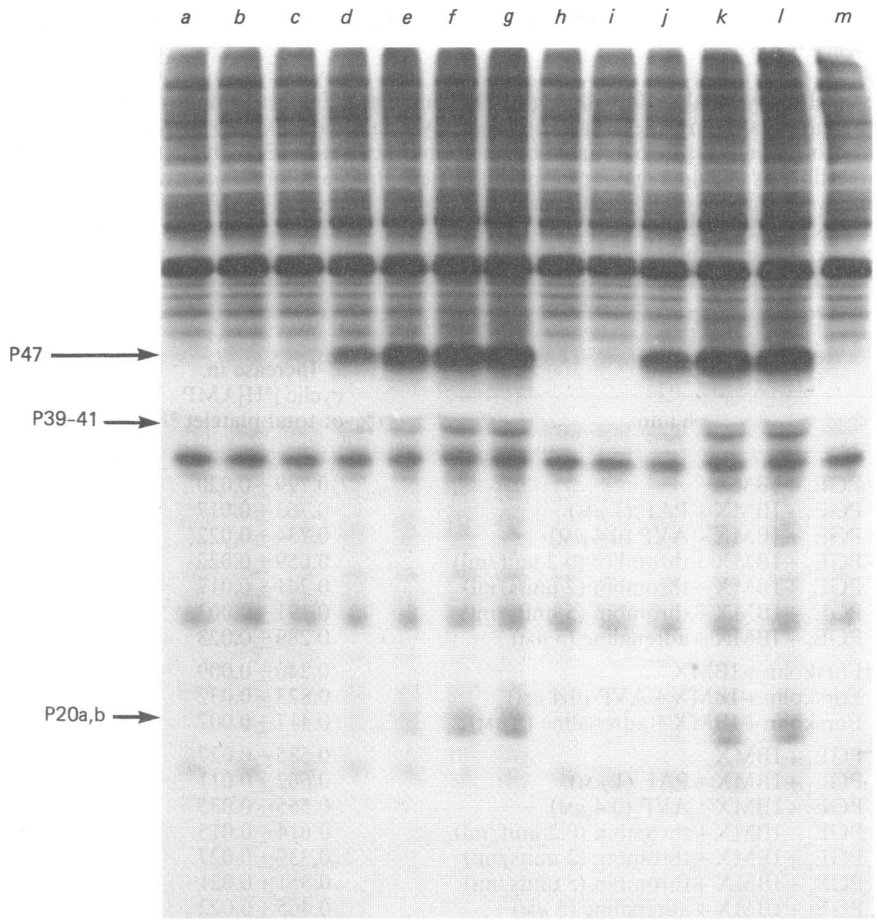

Fig. 5. Effects of different concentrations of PMA and diC_8 on the phosphorylation of polypeptides in washed platelets

This autoradiograph is from the experiment described in Fig. 4. Washed labelled platelets were incubated for 1 min with DMSO, diC_8 or PMA, and then for 0.5 min with 0.2 μM-PGE_1 and 1 mM-IBMX. Protein was precipitated with trichloroacetic acid, dissolved in electrophoresis sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis. Lanes are for the following treatments: (*a*) DMSO; (*b*) 0.4 μM-diC_8; (*c*) 1.0 μM-diC_8; (*d*) 4 μM-diC_8; (*e*) 10 μM-diC_8; (*f*) 40 μM-diC_8; (*g*) 100 μM-diC_8; (*h*) 0.4 nM-PMA; (*i*) 1.0 nM-PMA; (*j*) 10 nM-PMA; (*k*) 100 nM-PMA; (*l*) 1 μM-PMA; (*m*) 100 nM-phorbol.

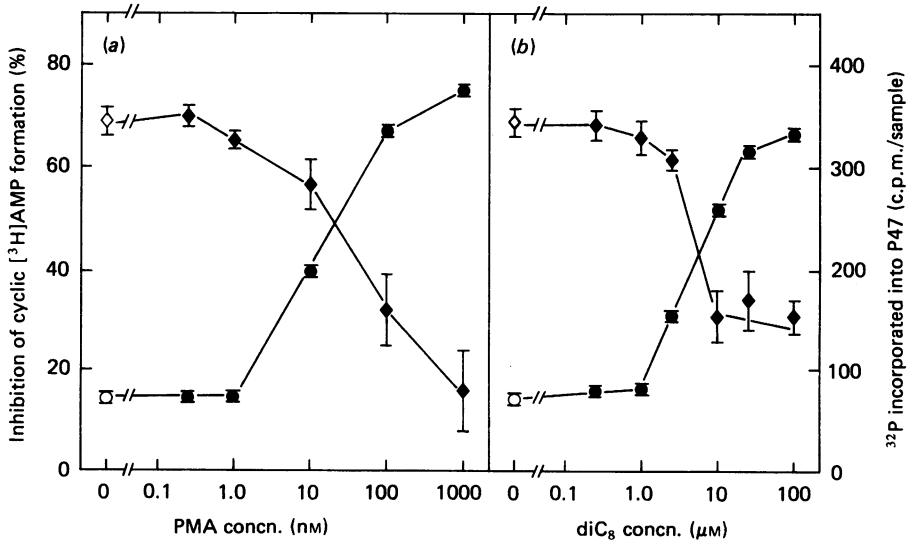

Fig. 6. Relationship between the activation of protein kinase C and inhibition of cyclic [^{3}H]AMP formation by adrenaline in platelets preincubated with PMA (*a*) or diC_8 (*b*)

The percentage inhibition of cyclic [^{3}H]AMP formation by 5 μM-adrenaline was calculated for the controls with DMSO (◇) and for each concentration of PMA or diC_8 added (◆) in the experiment shown in Fig. 4, and the corresponding values for the ^{32}P incorporated into P47 (○, ●) were determined as an index of the activation of protein kinase C in the same platelets. Values are means ± S.E. from triplicate incubations.

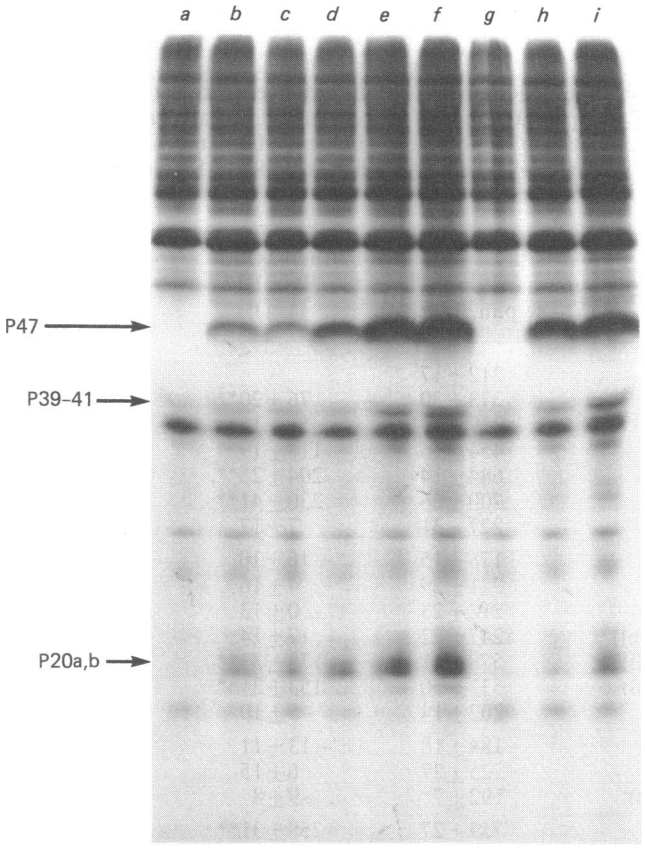

Fig. 7. Comparison of the effects of different aggregating agents and PMA on the phosphorylation of polypeptides in washed human platelets in the absence of PGE_1 and IBMX

Platelets labelled with both [^{3}H]adenine and [^{32}P]P_i were incubated for 5 min with indomethacin, phosphocreatine and creatine kinase, followed by 1 min with DMSO or PMA, as described in the Experimental section. Aggregating agents were then added and the incubations were terminated after 0.5 min by addition of trichloroacetic acid. Precipitated protein was analysed by SDS/polyacrylamide-gel electrophoresis, and an autoradiograph of the dried gel is shown. Lanes are for the following treatments: (*a*) DMSO; (*b*) 1 μM-PAF; (*c*) 0.4 μM-AVP; (*d*) 0.2 unit of thrombin/ml; (*e*) 2 units of thrombin/ml; (*f*) 5 units of thrombin/ml; (*g*) 5 μM-adrenaline (with 10 μM-propranolol); (*h*) 10 nM-PMA; (*i*) 100 nM-PMA.

However, in the present study, a role for ADP in the inhibition of adenylate cyclase by PMA was excluded by addition of sufficient phosphocreatine and creatine kinase to decrease the inhibitory effect of a high concentration of exogenous ADP to a very low value. Moreover, in a separate investigation in which the platelet dense granules were labelled with 5-hydroxy-[^{14}C]tryptamine, no ^{14}C was secreted in response to PMA under the present incubation conditions (R. J. Haslam & M. M. L. Davidson, unpublished work). The discrepancy between our results and the earlier study [36] may be accounted for by major differences in the experimental conditions: Ashby *et al.* [36] preincubated platelets with PMA for 10 min rather than 1 min, allowing much more time for secretion of ADP, and then incubated the platelets for a further 5 min with prostacyclin or forskolin in the absence of any inhibitor of cyclic AMP phosphodiesterase.

Our observation that PMA inhibited adenylate cyclase in intact platelets is difficult to reconcile with the enhanced responsiveness of the enzyme to PGE_1 or forskolin in the presence of GTP, demonstrated by Jakobs *et al.* [34] in membranes isolated from PMA-treated platelets. These workers attributed the effects they obtained to suppression of the inhibitory action of GTP, as a result of phosphorylation of the α_i-subunit of G_i by protein kinase C [37,48]. As treatment of platelets with PMA did not block the inhibition of membrane adenylate cyclase by a stable GTP analogue, phosphorylation of α_i may normally enhance its GTPase activity and facilitate its reassociation with the inhibitory $\beta\gamma$-subunit [49]. Such a mechanism could lead to an increase, but not a decrease, in hormone-stimulated cyclic AMP formation, as has been observed in S49 lymphoma cells [33,50]. It follows that, in intact platelets, G_i probably does not exert the tonic inhibitory effect on adenylate cyclase observed in isolated membranes and that PMA is likely to inhibit cyclic AMP formation by effects on components of the adenylate cyclase system other than G_i. Inhibition of the action of forskolin by PMA suggests that the latter affects the function of the catalytic subunit of adenylate cyclase or the stimulatory guanine-nucleotide-binding protein, G_s, which is required for an optimal forskolin effect [51]. The same mechanism could account for the initial inhibition of the effect of PGE_1, but the more potent action of PMA on the activation of adenylate cyclase by PGD_2 suggests an additional effect, perhaps at the receptor level, as reported for the β-adrenergic receptor in various cell types [29,30]. The failure of Jakobs *et al.* [34,37,48] to detect any inhibitory effects of PMA or protein kinase C on the adenylate cyclase activities of isolated platelet membranes could reflect selective dephosphorylation of protein kinase C substrates, loss of unidentified soluble factors or a dominant effect of phosphorylation of G_i.

The transient peak in platelet cyclic AMP content observed after addition of PGE_1 is a well-documented phenomenon (e.g. [10]). This effect is not observed with PGD_2 [52] or prostacyclin (W. Murphy & R. J. Haslam, unpublished work), and is therefore agonist-specific and independent of cyclic AMP formation. Previous work [10] and measurements of the initial rates of cyclic [^{3}H]AMP accumulation in the presence of IBMX in this study suggest that the activation of adenylate cyclase rapidly falls after addition of PGE_1. PMA blocked this decrease in adenylate cyclase activity in addition to inhibiting the immediate activation of the enzyme by PGE_1. The net effect at later incubation times was an increase in cyclic [^{3}H]AMP formation. Somewhat similar observations have been made in hepatocytes, in which PMA blocked both the activation of adenylate cyclase by glucagon and glucagon desensitization [28,53]. Although there is no evidence that PGE_1 can act on a second receptor to stimulate protein kinase C, as reported for glucagon [54], it is conceivable that a second receptor inhibits adenylate cyclase by another mechanism.

In contrast with the effects of PMA on the activation of adenylate cyclase in intact platelets, the attenuation of the inhibitory effects of adrenaline and thrombin is entirely consistent with previous observations on isolated membranes [34,48], and may be accounted for by the phosphorylation of the α_i-subunit of G_i. However, the

Table 3. Effects of different aggregating agents and of PMA on the activation of protein kinase C in intact human platelets in the absence and presence of increased cyclic AMP

These results are from the same experiment as that reported in Table 2, in which further details are given. Platelet protein was analysed by SDS/polyacrylamide-gel electrophoresis and the ^{32}P incorporated into P47 determined, as a measure of the activation of protein kinase C. Values are means ± S.E.M. from three separate incubations. The percentage increases in ^{32}P in P47 relative to the control with no additions other than DMSO are also given (means ± S.E. of the quotient). Significant effects are indicated (*$P < 0.05$; **$P < 0.01$, unpaired t test).

Additions		^{32}P in P47 (c.p.m./sample)	Increase in ^{32}P in P47 (%)
5 min	6 min		
DMSO	None	212 ± 17	–
	PAF (1 μM)	373 ± 30	76 ± 20**
	AVP (0.4 μM)	319 ± 19	51 ± 15*
	Thrombin (0.2 unit/ml)	454 ± 16	114 ± 19**
	Thrombin (2 units/ml)	644 ± 14	204 ± 25**
	Thrombin (5 units/ml)	700 ± 66	230 ± 41**
	Adrenaline (5 μM)	227 ± 31	7 ± 17
	PGE_1 + IBMX	178 ± 15	−16 ± 10
	PGE_1 + IBMX + PAF (1 μM)	221 ± 29	4 ± 16
	PGE_1 + IBMX + AVP (0.4 μM)	212 ± 23	0 ± 13
	PGE_1 + IBMX + thrombin (0.2 unit/ml)	247 ± 22	17 ± 14
	PGE_1 + IBMX + thrombin (2 units/ml)	578 ± 51	173 ± 33**
	PGE_1 + IBMX + thrombin (5 units/ml)	537 ± 50	153 ± 31**
	PGE_1 + IBMX + adrenaline (5 μM)	202 ± 14	−5 ± 10
	Forskolin + IBMX	184 ± 18	−13 ± 11
	Forskolin + IBMX + AVP (0.4 μM)	225 ± 27	6 ± 15
	Forskolin + IBMX + adrenaline (5 μM)	192 ± 7	−9 ± 8
PMA	None	759 ± 27	258 ± 31**
	PGE_1 + IBMX	684 ± 12	223 ± 26**
	Forskolin + IBMX	576 ± 44	172 ± 30**

relative failure of PMA to block the inhibition of cyclic [^{3}H]AMP formation caused by ADP is anomalous. The inhibition of platelet membrane adenylate cyclase by ADP has been shown to be GTP-dependent [11,55], but the effect of pertussis toxin on this is unknown. Our results suggest that the mechanism of inhibition of platelet adenylate cyclase by ADP may differ from that utilized by adrenaline.

The effects of PMA on both the stimulation of cyclic AMP formation by PGE_1 and the inhibition of this by adrenaline were reproduced by diC_8, but not by the biologically inactive PMA analogues, phorbol and 4α-PDD. Moreover, a close relationship was found between these effects and the phosphorylation of the 47 kDa substrate of protein kinase C (P47). These observations indicate that protein kinase C mediates these actions of PMA, though there is no reason to believe that P47 itself affects adenylate cyclase activity. Studies on the effects of PMA on protein phosphorylation in intact ^{32}P-labelled platelets have identified many other substrates of protein kinase C, including myosin light chain [47] and various transmembrane glycoproteins [56,57]. In the present study, we have noted major increases in ^{32}P incorporation into at least two polypeptides in the 39–41 kDa range (P39–41). These may include the 41 kDa α_i-subunit of G_i, which is phosphorylated on incubation of platelet membranes with protein kinase C [37]. In contrast with the above findings, the weak stimulation of cyclic [^{3}H]AMP formation by diC_8 concentrations below those causing inhibition was not associated with phosphorylation of platelet proteins and is unlikely to be mediated by protein kinase C. Ashby *et al.* [36] observed a more marked stimulation of cyclic AMP formation by 1-oleoyl-2-acetylglycerol, which may represent the same phenomenon.

The results confirm our previous observations that AVP and PAF, which inhibit adenylate cyclase in isolated platelet membranes, do not do so in intact platelets [14,17]. However, the explanation of these findings offered by Jakobs *et al.* [34], namely that these agonists activate protein kinase C, and thus impair signal transduction by G_i, is not supported by our results. Thus these agonists were only weak activators of protein kinase C under experimental conditions that prevented the formation or action of secondary mediators, such as thromboxane A_2 and ADP. Moreover, in the presence of PGE_1, which is necessary for detection of any inhibition of cyclic AMP formation, AVP and PAF were unable to activate protein kinase C at all. This reflects the well-documented inhibition of phospholipase C activation by agents that increase platelet cyclic AMP [1]. More complex results were obtained with thrombin. A low concentration (0.2 unit/ml) that causes an optimal GTP-dependent inhibition of adenylate cyclase in membrane preparations [19] behaved identically with AVP and PAF and did not inhibit cyclic [^{3}H]AMP formation in intact platelets or activate protein kinase C in the presence of PGE_1. Higher thrombin concentrations both inhibited cyclic [^{3}H]AMP formation and activated protein kinase C. Our results suggest that this inhibition of adenylate cyclase could consist of two components.

First, thrombin may exert a direct effect through G_i [19], though this may be partially inhibited by the simultaneous activation of protein kinase C [37], and second, thrombin may inhibit adenylate cyclase through protein kinase C. This would explain why PMA was less effective in blocking inhibition caused by thrombin than that caused by adrenaline.

We have previously noted that many agonists that activate platelet phospholipase C seem unable to inhibit adenylate cyclase in intact human platelets, despite their ability to do so in membrane preparations [17]. The present results show that this generalization breaks down in the case of high, though not low, thrombin concentrations, suggesting that the strength of the stimulus may be an important factor in determining the effector system that is activated. In a study by McGowan & Detwiler [20], this difference was not observed, but chymotrypsin treatment of platelets was found to block the inhibition of cyclic AMP formation by thrombin, but not the activation of phospholipase C or protein kinase C. Moreover, γ-thrombin exerted the same effects as we obtained with low concentrations of α-thrombin. As discussed previously [17], at least two explanations of our results and of the similar findings of McGowan & Detwiler [20] are possible. Distinct receptor subtypes could mediate the stimulation of phospholipase C and inhibition of adenylate cyclase, as suggested for thrombin [20], in which case a mechanism must exist for the selective inactivation of the latter pathway in intact platelets. Alternatively, a single receptor type could interact preferentially with the adenylate cyclase effector system in isolated membranes and with the phospholipase C effector system in the intact cells. There is increasing support for the view that different guanine-nucleotide-binding proteins mediate the inhibition of adenylate cyclase and activation of phospholipase C in platelets [58–60], but little compelling evidence for distinct receptor subtypes that could mediate these effects [14,17,20]. We therefore propose that, in the intact platelet, the receptors for PAF, AVP and thrombin may interact preferentially with the G-protein that activates phospholipase C and that only when large numbers of receptors are occupied by a full agonist is G_i also activated. This hypothesis is consistent with our present observations and could explain why high thrombin concentrations, but neither low concentrations nor γ-thrombin, can inhibit adenylate cyclase in intact human platelets. This view is also supported by the finding that PAF does inhibit adenylate cyclase in intact rabbit platelets [61], which possess many more PAF receptors than do human platelets [62].

This work was supported by a grant (MT-5626) from the Medical Research Council of Canada.

REFERENCES

1. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
2. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
3. Rink, T. J. & Hallam, T. J. (1984) Trends Biochem. Sci. **9**, 215–219
4. Lapetina, E. G. (1983) Life Sci. **32**, 2069–2082
5. MacIntyre, D. E., Pollock, W. K., Shaw, A. M., Bushfield, M., MacMillan, L. J. & McNicol, A. (1985) in Mechanisms of Stimulus–Response Coupling in Platelets (Westwick, J., Scully, M. F., MacIntyre, D. E. & Kakkar, V. V., eds.), pp. 127–144, Plenum Press, New York
6. Fisher, G. J., Bakshian, S. & Baldassare, J. J. (1985) Biochem. Biophys. Res. Commun. **129**, 958–964
7. Siess, W., Weber, C. & Lapetina, E. G. (1984) J. Biol. Chem. **259**, 8286–8292
8. Hallam, T. J. & Rink, T. J. (1985) FEBS Lett. **186**, 175–179
9. Sweatt, J. D., Blair, I. A., Cragoe, E. J. & Limbird, L. E. (1986) J. Biol. Chem. **261**, 8660–8666
10. Haslam, R. J. (1973) Ser. Haematol. **6**, 333–350
11. Cooper, D. M. F. & Rodbell, M. (1979) Nature (London) **282**, 517–518
12. Jakobs, K. H., Saur, W. & Schultz, G. (1978) FEBS Lett. **85**, 167–170
13. Haslam, R. J., Davidson, M. M. L. & Desjardins, J. V. (1978) Biochem. J. **176**, 83–95
14. Vanderwel, M., Lum, D. S. & Haslam, R. J. (1983) FEBS Lett. **164**, 340–344
15. Williams, K. A. & Haslam, R. J. (1984) Biochim. Biophys. Acta **770**, 216–223
16. Avdonin, P. V., Svitina-Ulitina, I. V., Letin, V. L. & Tkachuk, V. A. (1985) Thromb. Res. **40**, 101–112
17. Haslam, R. J., Williams, K. A. & Davidson, M. M. L. (1985) in Mechanisms of Stimulus–Response Coupling in Platelets (Westwick, J., Scully, M. F., MacIntyre, D. E. & Kakkar, V. V., eds.), pp. 265–280, Plenum Press, New York
18. Best, L. C., McGuire, M. B., Martin, T. J., Preston, F. E. & Russell, R. G. G. (1979) Biochim. Biophys. Acta **583**, 344–351
19. Aktories, K. & Jakobs, K. H. (1984) Eur. J. Biochem. **145**, 333–338
20. McGowan, E. B. & Detwiler, T. C. (1986) J. Biol. Chem. **261**, 739–746
21. Drummond, A. H. & MacIntyre, D. E. (1985) Trends Pharmacol. Sci. **6**, 233–234
22. Nishizuka, Y. (1986) Science **233**, 305–312
23. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851
24. MacIntyre, D. E., McNichol, A. & Drummond, A. H. (1985) FEBS Lett. **180**, 160–164
25. Watson, S. P. & Lapetina, E. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2623–2626
26. Zavoico, G. B., Halenda, S. P., Sha'afi, R. I. & Feinstein, M. B. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3859–3862
27. Garte, S. J. & Belman, S. (1980) Nature (London) **284**, 171–173
28. Heyworth, C. M., Whetton, A. D., Kinsella, A. R. & Houslay, M. D. (1984) FEBS Lett. **170**, 38–42
29. Kelleher, D. J., Pessin, J. E., Ruoho, A. E. & Johnson, G. L. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4316–4320
30. Kassis, S., Zaremba, T., Patel, J. & Fishman, P. H. (1985) J. Biol. Chem. **260**, 8911–8917
31. Quilliam, L. A., Dobson, P. R. M. & Brown, B. L. (1985) Biochem. Biophys. Res. Commun. **129**, 898–903
32. Sugden, D., Vanecek, J., Klein, D. C., Thomas, T. P. & Anderson, W. B. (1985) Nature (London) **314**, 359–361
33. Bell, J. D., Buxton, I. L. O. & Brunton, L. L. (1985) J. Biol. Chem. **260**, 2625–2628
34. Jakobs, K. H., Bauer, S. & Watanabe, Y. (1985) Eur. J. Biochem. **151**, 425–430
35. Zucker, M. B., Troll, W. & Belman, S. (1974) J. Cell Biol. **60**, 325–336
36. Ashby, B., Kowalska, M. A., Wernick, E., Rigmaiden, M., Daniel, J. L. & Smith, J. B. (1985) J. Cyclic Nucleotide Protein Phosphorylation Res. **10**, 473–483
37. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) Eur. J. Biochem. **151**, 431–437
38. Williams, K. A. & Haslam, R. J. (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 1522
39. Imaoka, T., Lynham, J. A. & Haslam, R. J. (1983) J. Biol. Chem. **258**, 11404–11414

40. Molnar, J. & Lorand, L. (1961) Arch. Biochem. Biophys. **93**, 353–363
41. Mustard, J. F., Perry, D. W., Ardlie, N. G. & Packham, M. A. (1972) Br. J. Haematol. **22**, 193–204
42. Aster, R. H. & Jandl, J. H. (1964) J. Clin. Invest. **43**, 843–855
43. Haslam, R. J. & McClenaghan, M. D. (1981) Nature (London) **292**, 364–366
44. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
45. Haslam, R. J., Lynham, J. A. & Fox, J. E. B. (1979) Biochem. J. **178**, 397–406
46. Lapetina, E. G., Reep, B., Ganong, B. R. & Bell, R. M. (1985) J. Biol. Chem. **260**, 1358–1361
47. Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) Nature (London) **306**, 490–492
48. Watanabe, Y., Horn, F., Bauer, S. & Jakobs, K. H. (1985) FEBS Lett. **192**, 23–27
49. Bauer, S. & Jakobs, K. H. (1986) FEBS Lett. **198**, 43–46
50. Bell, J. D. & Brunton, L. L. (1986) J. Biol. Chem. **261**, 12036–12041
51. Daly, J. W. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **17**, 81–89
52. Mills, D. C. B. & Macfarlane, D. E. (1974) Thromb. Res. **5**, 401–412
53. Heyworth, C. M., Wilson, S. P., Gawler, D. J. & Houslay, M. D. (1985) FEBS Lett. **187**, 196–200
54. Wakelam, M. J. O., Murphy, G. J., Hruby, V. J. & Houslay, M. D. (1986) Nature (London) **323**, 68–71
55. Mellwig, K. P. & Jakobs, K. H. (1980) Thromb. Res. **18**, 7–17
56. Feuerstein, N., Monos, D. S. & Cooper, H. L. (1985) Biochem. Biophys. Res. Commun. **126**, 206–213
57. Bourguignon, L. Y. W., Walker, G. & Bourguignon, G. J. (1985) J. Biol. Chem. **260**, 11775–11780
58. Haslam, R. J. & Davidson, M. M. L. (1984) J. Receptor Res. **4**, 605–629
59. Houslay, M. D., Bojanic, D. & Wilson, A. (1986) Biochem. J. **234**, 737–740
60. Grandt, R., Aktories, K. & Jakobs, K. H. (1986) Biochem. J. **237**, 669–674
61. Haslam, R. J. & Vanderwel, M. (1982) J. Biol. Chem. **257**, 6879–6885
62. Hwang, S.-B., Lee, C.-S. C., Cheah, M. J. & Shen, T. Y. (1983) Biochemistry **22**, 4756–4763

Received 17 October 1986/22 December 1986; accepted 14 January 1987