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Effects of protein kinase C activation on human platelet cyclic AMP metabolism

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Treatment of intact human platelets with the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), specifically inhibited PGD_2-induced cyclic AMP formation without affecting the regulation of cyclic AMP metabolism by PGI_2, PGE_1, 6-keto-PGE_1, adenosine or adrenaline. This action of PMA was: (i) concentration-dependent; (ii) not mediated by evoked formation or release of endogenous regulators of adenylate cyclase activity (thromboxane A_2 or ADP); (iii) mimicked by 1,2-dioctanoylglycerol (DiC_8) but not by 4α-phorbol 12,13-didecanoate (which does not activate protein kinase C); (iv) attenuated by Staurosporine. These results indicate that activation of protein kinase C in platelets may provide a regulatory mechanism to abrogate the effects of the endogenous adenylate cyclase stimulant PGD_2 without compromising the effects of exogenous stimulants of adenylate cyclase (PGI_2, 6-keto-PGE_1, adenosine).

Platelet; cyclic AMP; Protein kinase C

1. INTRODUCTION

Platelet reactivity is controlled by the complex interactions of at least two different intracellular second messenger systems. Inhibition of platelet responsiveness is mediated by agonist (e.g. PGI_2, PGD_2 or adenosine)-induced elevation of intracellular cyclic AMP concentration [1]. Platelet activation in response to many agonists (e.g. thrombin, vasopressin, platelet-activating factor and thromboxane (TXA_2)) is mediated via elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and formation of 1,2-diacylglycerol (DG) [2,3]: both events are possible sequelae of agonist-induced phosphoinositide hydrolysis [4]. There is much evidence that other agonists (e.g. adrenaline, ADP) that induce platelet activation inhibit adenylate cyclase in platelet lysates, an effect which, in intact platelets, is manifest as inhibition of agonist-induced cyclic AMP formation [1]. However, a cause and effect relationship between inhibition of adenylate cyclase and promotion of platelet activation remains to be established. Many of the effects of these intracellular messengers are mediated via activation of specific protein kinase enzymes; calcium/calmodulin-dependent protein kinases; DG-dependent protein kinase C and cyclic AMP-dependent protein kinase [4].

Besides its role in promoting platelet activation, a number of recent studies have indicated that activated protein kinase C may also subserve a negative feedback role to reverse or inhibit agonist-induced phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ in platelets [5] and other cell types [6]. Moreover, in other cells, activation of protein

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kinase C by tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) also modulates cellular cyclic AMP metabolism. Indeed, phorbol esters have been reported either to enhance basal or hormone-elevated cyclic AMP levels [7] or to inhibit agonist-induced elevations in the cellular cyclic AMP content [8].

These actions of protein kinase C may be mediated by effects on the hormone receptor [9]; the guanine nucleotide-binding regulatory proteins, N_s/G_s [10] or N_i/G_i [11,12]; the catalytic subunit of adenylate cyclase [13] and/or cyclic nucleotide phosphodiesterase [14]. Moreover, protein kinase C may mediate complex regulation of cyclic AMP metabolism by acting at different sites within a single cell type [15]. Here, we have investigated the effects of protein kinase C activation on regulation of cyclic AMP metabolism in intact human platelets by agonists that act upon distinct receptors which are coupled via N_s (e.g. PGD_2, PGI_2, adenosine) or N_i (e.g. α_2-adrenoreceptors), respectively, to stimulation or inhibition of adenylate cyclase.

2. EXPERIMENTAL

Blood was obtained and plasma-free platelet suspensions (2×10^8 platelets per ml) were prepared by gel filtration as described [16]. Cyclic AMP was extracted from platelets using ethanol. The extracts were evaporated to dryness (60°C), reconstituted in acetate buffer (50 mM, pH 5) and the cyclic AMP content was estimated by radioimmunoassay using iodinated tracers [16].

Adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3′-[^{125}I]iodotyrosine methyl ester (600 Ci per mmol) was obtained from Amersham International (Amersham, England). PGD_2, PGI_2, PGE_1 and 6-keto-PGE_1 were obtained from Upjohn (Kalamazoo, MI). 1,2-Dioctanoylglycerol (DiC_8) was obtained from Avanti Polar Lipids (Birmingham, AL) and was dissolved in ethanol. Other reagents were obtained from Sigma (England); PMA and 4α-phorbol 12,13-didecanoate (4α-PDD) were dissolved in DMSO and other reagents were dissolved in iso-osmotic saline. All incubations (37°C) were performed in triplicate and experiments were repeated at least three times using blood obtained from different donors.

3. RESULTS AND DISCUSSION

Incubation (4 min, 37°C) with PMA ($\leqslant$300 nM) did not alter the level of cyclic AMP in control (7.9 ± 0.3 pmol/10^8 platelets) or isobutylmethylxanthine (IBMX; 1 mM, 30–60 min)-treated platelets (19.9 ± 2.3 pmol/10^8 platelets). Incubation (2 min, 37°C) with PGD_2 ($\leqslant 3\,\mu$M), PGI_2 ($\leqslant$300 nM), PGE_1 ($\leqslant 10\,\mu$M), 6-keto-PGE_1 ($\leqslant 10\,\mu$M) or adenosine ($\leqslant 100\,\mu$M) resulted in concentration-dependent elevations in platelet

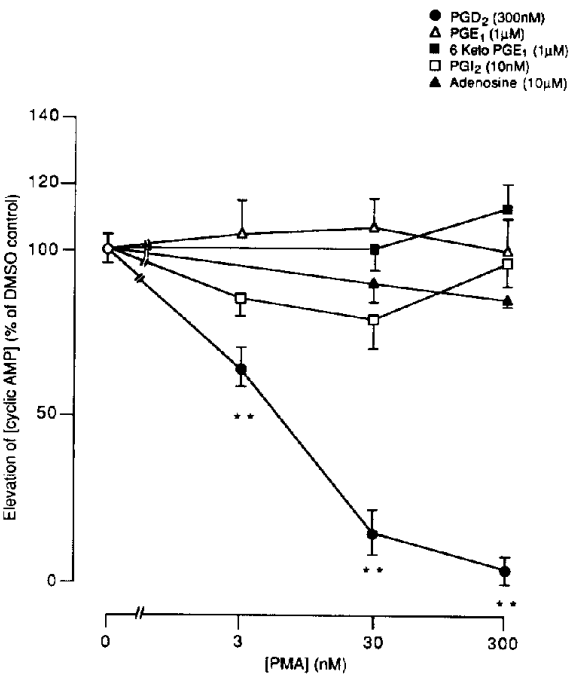

Fig.1. Effects of PMA on agonist-induced elevations in platelet cyclic AMP content. Gel-filtered platelet samples (0.4 ml, 2×10^8 platelets per ml) were incubated (2 min) with the concentrations of PMA indicated before addition of submaximal concentrations of PGD_2 (300 nM) (●); PGE_1 (1 μM) (△); 6-keto-PGE_1 (1 μM) (■); PGI_2 (100 nM) (□) or adenosine (10 μM) (▲) for a further 2 min. Cyclic AMP content was estimated as described in section 2. The basal cyclic AMP content was 8 ± 0.3 pmol per 10^8 platelets (n = 12) and the control elevations (in the presence of 0.15% DMSO) were as follows: PGD_2, 81 ± 4, n = 7; PGE_1, 18 ± 4.2, n = 3; 6-keto-PGE_1, 11.5 ± 1.8, n = 3; PGI_2, 120 ± 9.2, n = 3; adenosine, 5.3 ± 0.4, n = 3 (all expressed as pmol per 10^8 platelets above the basal level). Data were compared to the DMSO control using Student's t-test: $^{**}P <$ 0.001.

cyclic AMP content (not shown). Submaximal concentrations of each compound (inducing between 40 and 60% of maximal elevation in platelet cyclic AMP content) were selected in order to examine the effects of PMA on agonist-induced cyclic AMP formation. Preincubation (2 min) with PMA (3–300 nM) resulted in concentration-dependent inhibition of the rise in platelet cyclic AMP content induced by submaximal concentrations of PGD_2 (300 nM) but not by PGI_2 (100 nM), PGE_1 (1 μM), 6-keto-PGE_1 (1 μM) or adenosine (1 μM) (fig.1). The inhibitory effect of PMA on PGD_2-induced cyclic AMP formation (I_{50} = 7.5 nM) was shared by 1,2-dioctanoylglycerol (I_{50} = 30 μM), but not by the non-tumour-promoting phorbol ester 4α-PDD. Moreover these effects of PMA and DiC_8 were suppressed by Staurosporine (100 nM), an agent that inhibits protein kinase C [17] and impairs PMA- and DiC_8-induced phosphorylation of the 40 kDa protein, the major substrate for platelet protein kinase C (not shown). Collectively, these observations indicate that selective inhibition of PGD_2-induced cAMP formation by PMA and DiC_8 is consistent with, and may be attributed to, the activation of protein kinase C.

As PMA is known to induce platelet activation, degranulation and eicosanoid biosynthesis, we investigated whether the effects of PMA on PGD_2-induced cyclic AMP formation could be mediated by agents (e.g. ADP, TXA_2) released by platelets and which have been reported to inhibit adenylate cyclase [1,5]. In the presence of flurbiprofen (10 μM), which maximally inhibits prostanoid (TXA_2) biosynthesis, PMA inhibited PGD_2-induced cyclic AMP formation (I_{50} = 10 ±

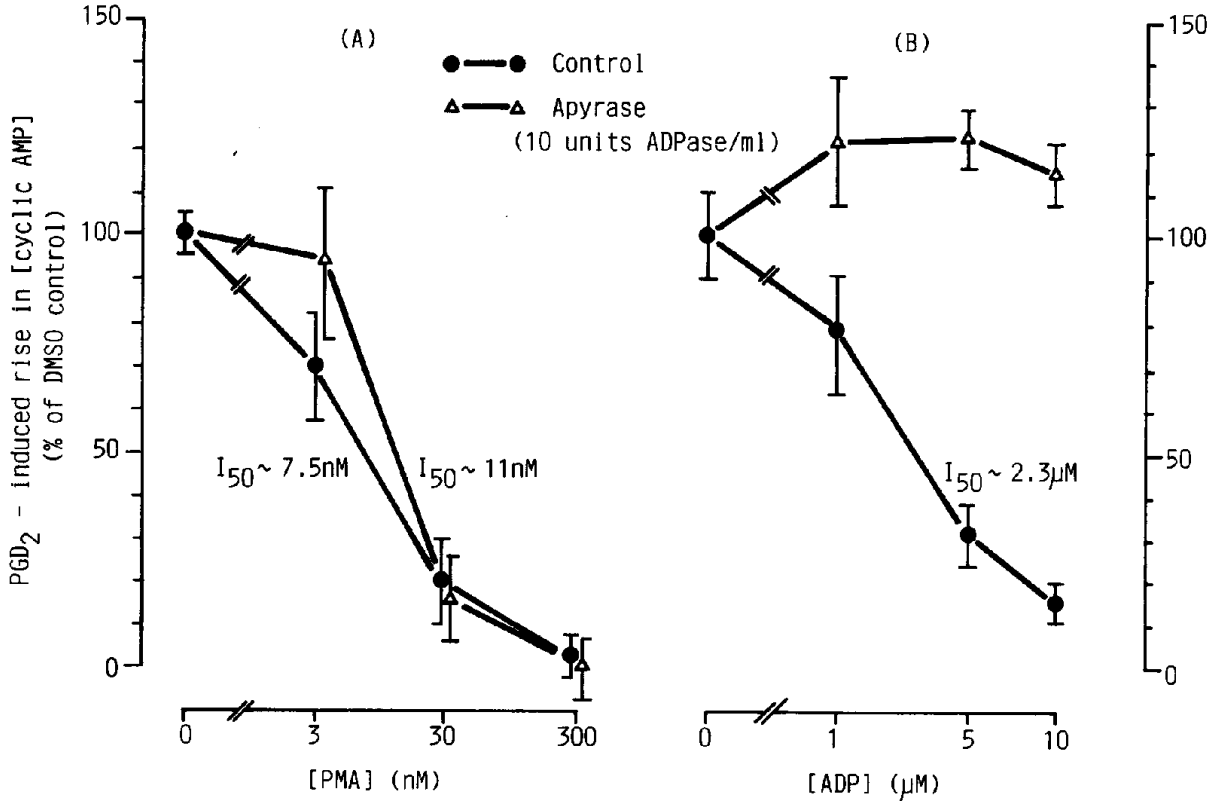

Fig.2. Effects of apyrase on the inhibition of PGD_2-induced elevation of platelet cyclic AMP concentration by (A) PMA and (B) ADP. Control (●) and apyrase (10 U ADPase per ml; 10 min)-treated (△) platelet samples were incubated for 2 min with (A) PMA or (B) ADP at the concentrations indicated prior to the addition of PGD_2 (300 nM) for a further 2 min and cyclic AMP content was monitored as described in section 2. Data are means ± SE from three different experiments.

1.5 nM), indicating that this effect is not mediated by endogenous TXA_2.

The role of PMA-induced ADP release in blocking PGD_2-induced cyclic AMP formation was investigated by treating platelets with the ADP scavengers apyrase or creatine phosphate (2 mM) plus creatine phosphokinase (50 U/ml). Under conditions where the inhibition of PGD_2-induced cyclic AMP formation by exogenously added ADP (1–10 μM) was abolished, apyrase had no significant effect on the inhibition by PMA (I_{50} = 11 nM, fig.2). Similarly, this effect of PMA was not altered by incubation (10–40 min) with creatine phosphate plus creatine phosphokinase (not shown). Thus, PMA-induced inhibition of the rise in cyclic AMP content cannot be attributed to formation or release of endogenous inhibitors of adenylate cyclase.

Activation of cyclic nucleotide phosphodiesterase by protein kinase C could contribute, at least in part, to the actions of PMA. Accordingly, the effects of PMA on PGD_2-induced cyclic AMP formation were examined in the presence of the phosphodiesterase inhibitor IBMX. Incubation (15–60 min) with IBMX (1 mM) resulted in a doubling of the PGD_2-induced rise in cyclic AMP content but only marginally inhibited the effect of PMA (fig.3). Hence, the effect of PMA is most likely due to inhibition of PGD_2-induced cyclic AMP formation although augmentation of phosphodiesterase activity may contribute.

The specificity of this action of PMA suggests that it is mediated via protein kinase C-dependent phosphorylation of the PGD_2 receptor rather than via an effect on the 'G-proteins' or the adenylate cyclase catalytic subunit. In agreement with this, Ashby et al. [18] reported that neither PMA nor the synthetic diglyceride oleoylacetylglycerol had any direct effect on PGI_2- or forskolin-induced cyclic AMP formation. Such inhibitory receptor phosphorylations have been demonstrated using a number of different receptors and cell systems [19].

The potential physiological role of this phenomenon is questionable since there is no evidence that in intact platelets agents (such as thrombin, vasopressin or platelet-activating factor) which activate protein kinase C, via diacylglycerol formation, have any effect on PGD_2-induced cyclic AMP formation. However,

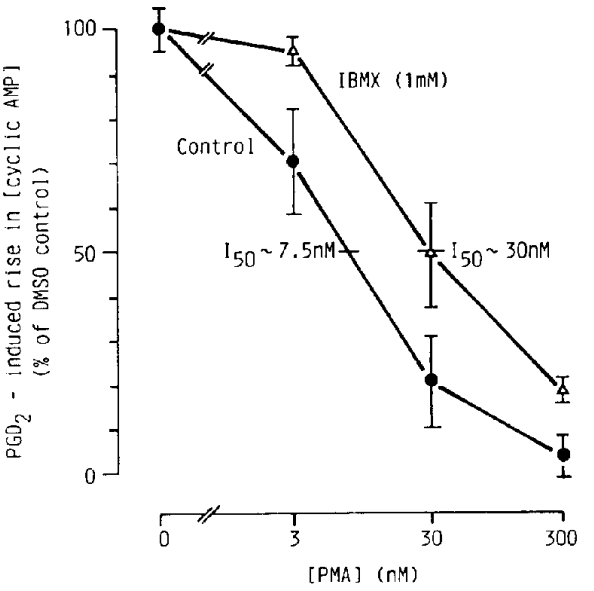

Fig.3. Effect of IBMX on the inhibition of PGD_2-induced elevation in cyclic AMP content by PMA. Control (●) and IBMX (1 mM, 20–50 min)-treated platelets (△) were incubated with PMA (2 min) at the concentrations indicated before addition of PGD_2 (300 nM) for a further 2 min and cyclic AMP content was monitored as described in section 2. The PGD_2-induced rise in cyclic AMP content (control 115 ± 10 pmol per 10^8 cells; n = 3) was approximately doubled in the presence of IBMX (222 ± 12 pmol per 10^8 cells, n = 3). Data are means ± SE from three different experiments.

the possibility remains that, under certain pathophysiological conditions, activation of protein kinase C in platelets may act to abrogate the effects of the endogenous (i.e. platelet-derived) adenylate cyclase stimulant PGD_2, without compromising the actions of naturally occurring exogenous stimulants of adenylate cyclase (e.g. PGI_2, 6-keto-PGE_1 or adenosine). Indeed, it has been noted that platelets from patients with myeloproliferative disorders or acute thrombotic episodes show a specific loss of PGD_2-mediated control of adenylate cyclase activity whilst retaining normal PGI_2 and PGE_1 responsiveness [19]. The potential role of protein kinase C in these disorders is worthy of investigation.

The possible effects of protein kinase C on the inhibitory control of adenylate cyclase activity in

intact platelets were investigated by examining the effects of PMA on adrenaline-induced inhibition of platelet cyclic AMP formation. Platelet α_2-adrenoreceptors are coupled to inhibition of adenylate cyclase via the inhibitory G-protein N_i [1]. Addition of adrenaline (1–10 μM) to intact platelets resulted in concentration-dependent inhibition of the rises in cyclic AMP content induced by PGI_2 (100 nM), PGE_1 (1 μM) or PGD_2 (1 μM) (not shown). The inhibitory effects of adrenaline (1–5 μM) on PGE_1-induced elevations in cyclic AMP content were unaffected by pre-incubation with PMA ($\leqslant$1 μM) (fig.4).

Hence, in intact platelets, protein kinase C activation does not interfere with α_2-adrenoreceptor-dependent, N_i-mediated, inhibition of cyclic AMP formation. This observation contrasts with the reported blockade of N_i-mediated inhibition of adenylate cyclase activity in platelets and S49 lymphoma cells [11,12]. Whilst the reasons for this discrepancy are unknown, it may be explained by different experimental conditions: Jakobs and coworkers [11,12] measured cyclase activity in platelet membrane preparations whilst the present study was conducted using intact platelets. Indeed, studies in other cell types have revealed differences in the regulation of adenylate cyclase in lysates as compared to whole cells where there may be complex interactions between the different intracellular second messenger systems [20].

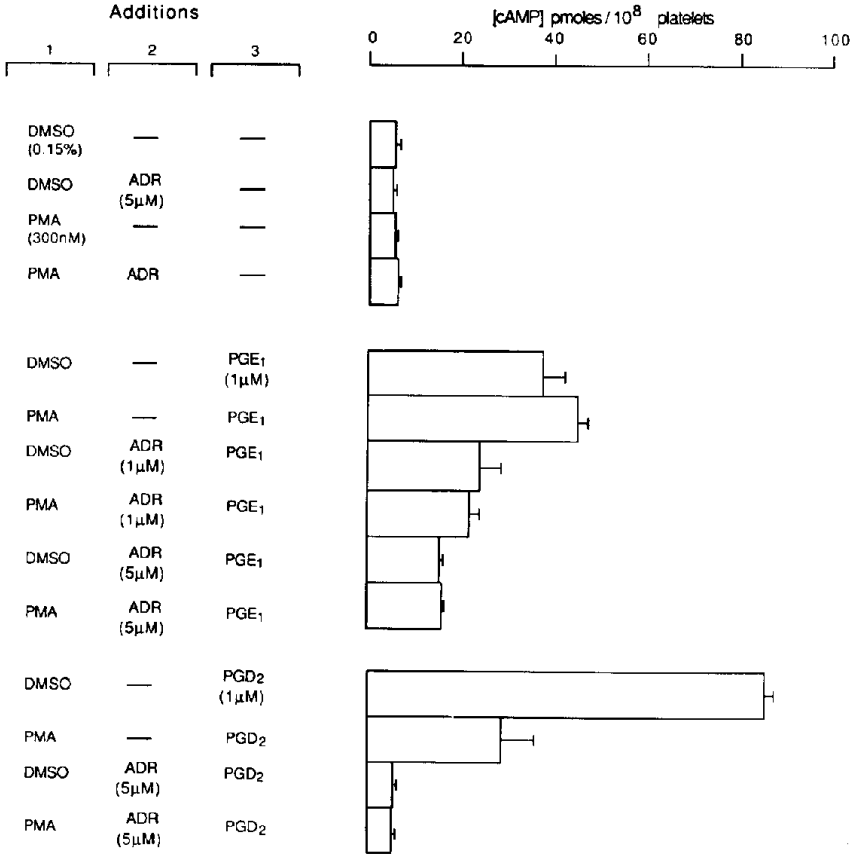

Fig.4. Effects of PMA on the modulation of platelet cyclic AMP content by PGE_1, PGD_2 and adrenaline. Where indicated, gel-filtered platelet samples were incubated with DMSO (0.15%, 4 min), PMA (300 nM, 4 min), adrenaline (1–5 μM, 2 min), PGE_1 (1 μM, 1 min) or PGD_2 (1 μM, 1 min) and cyclic AMP content was monitored as described in section 2. Data are means ± SE from a single experiment representative of three.

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