Tumour-promoting phorbol esters inhibit agonist-induced phosphatidate formation and Ca^{2+} flux in human platelets

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Tumour-promoting phorbol esters (phorbol-12-myristate-13-acetate, PMA; phorbol-12,13-dibutyrate, PDBu) but not 4β-phorbol, activate protein kinase C. Using human platelets pre-labelled with quin2 or $^{32}PO_4$ we examined the effects of these compounds on human platelet cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and on [^{32}P]phosphatidic acid ([^{32}P]PtdOH). PMA and PDBu, but not 4β-phorbol inhibited thrombin-, PAF- and vasopressin-induced elevation of $[Ca^{2+}]_i$ and [^{32}P]PtdOH formation. It is suggested that protein kinase C may act to terminate the transduction processes that link receptor occupancy to cellular activation.

Platelet *Phorbol ester* *Protein kinase C* *Polyphosphoinositide* *Calcium*

1. INTRODUCTION

In platelets, as in other cell types, agonist-receptor interaction at the plasma membrane modulates the levels of specific second messenger molecules that, in turn, influence the rates of those key biochemical reactions governing the cellular response. Inhibition of platelet responsiveness is associated with an elevation of the intracellular concentration of cAMP [1]. In contrast, stimulation of platelet responsiveness is associated with an elevation of the intracellular concentrations of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and 1,2-diacylglycerol (DAG) [2,3] as a consequence of a common receptor-operated transduction process: inositol phospholipid hydrolysis [4]. Agonist-induced hydrolysis of phosphatidylinositol (PtdIns), PtdIns4-phosphate (PtdIns4P) or, more likely, PtdIns4,5-bisphosphate (PtdIns4,5P_2) by phospholipase C yields DAG and the corresponding inositol phosphate(s). Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) can mobilise Ca^{2+} from intracellular (non-mitochondrial) stores [5], and phosphatidic acid (PtdOH) the immediate product of DAG metabolism, may exert Ca^{2+} ionophoric effects [6]. DAG, via activation of protein kinase C, and elevated $[Ca^{2+}]_i$, via activation of Ca^{2+}-calmodulin dependent protein kinase(s), may act independently or synergistically to induce platelet activation. The effects of endogenous DAG on protein kinase C and on cellular responsiveness can be mimicked by tumour promoting phorbol esters (e.g., phorbol-12-myristate-13-acetate, PMA; phorbol-12,13-dibutyrate, PDBu), but not by 4β-phorbol [7].

Although PMA influences phospholipid metabolism and agonist-induced elevation of $[Ca^{2+}]_i$ in neutrophils [8,9], its effects on inositol lipid metabolism and $[Ca^{2+}]_i$ in platelets have not been examined. We show here that PMA and PDBu, but not 4β-phorbol, impair the transduction processes, namely phosphoinositide metabolism and elevation of $[Ca^{2+}]_i$, that follow the combination of thrombin, vasopressin and PAF with their receptors on the platelet plasma membrane.

2. EXPERIMENTAL

Phospholipid studies were carried out on platelets isolated from fresh human blood essentially as previously described [10]. Briefly, platelets suspended in a Ca^{2+}-free, phosphate-free, Hepes buffer (pH 7.4) were incubated (37°C, 90 min)

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with carrier-free [^{32}P]orthophosphate (30 μCi/ml). Platelets were then pelleted, resuspended in 1.5 times the labelling volume of fresh buffer and aliquots (0.4 ml, 0.5–1 mg protein) were dispensed into plastic tubes at 37°C. PMA, PDBu, 4β-phorbol or dimethyl sulphoxide (DMSO) (as control) were added for 2 min and reactions were initiated by the addition of agonists: PAF (10 nM); vasopressin (100 nM); thrombin (0.1 U/ml). After 30 s reactions were terminated [10] and lipids were extracted, dried at 40°C under N_2, separated by 2-dimensional thin-layer chromatography [11] and visualized by exposure to iodine vapour. The spots corresponding to PtdOH were scraped into vials and counted for radioactivity in a liquid scintillation counter. Note that under the conditions of these experiments changes in [^{32}P]PtdOH reflect changes in PtdOH concentration [12].

Platelet cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was monitored essentially as described previously [13] using the fluorescent quinoline dye, quin2 [14]. Quin2-labelled platelets were separated from plasma containing extraneous dye by gel filtration on columns of Sepharose 2B equilibrated with a modified Hepes-buffered Tyrodes solution. The quin2 content of platelets was around 1 mmol/l cell H_2O. Gel-filtered platelets were stored at 37°C in stoppered plastic tubes, and the external free Ca^{2+} concentration was adjusted to 1 mM by addition of $CaCl_2$ immediately before use. Platelets (2 ml, ~5 × 10^7 cells/ml) were placed in 1 cm square quartz cuvettes at 37°C and fluorescence was monitored in a Perkin-Elmer LS3 Fluorescence Spectrometer at 37°C with standard monochromator settings of 339-nm excitation and 492-nm emission.

2.1. *Materials*

Thrombin, vasopressin, PMA, PDBu and 4β-phorbol were obtained from Sigma, London. PAF was purchased from Bachem, Bubbendorf, Switzerland and quin2-AM from Lancaster Synthesis, Morecambe, England. PMA, PDBu and 4β-phorbol were dissolved in DMSO.

3. RESULTS

4β-Phorbol or PMA ($\leqslant$1.6 μM) did not significantly alter the levels of [^{32}P]PtdOH in resting platelets. Thrombin (0.1 U/ml), vasopressin (100 nM) and PAF (10 nM) stimulated [^{32}P]PtdOH formation respectively 5.1 ± 0.9-fold, 2.5 ± 0.5-fold and 3.2 ± 0.7-fold (mean ± SE) over basal levels. Prior exposure of platelets to PMA (16 nM–1.6 μM) but not to 4β-phorbol ($\leqslant$1.6 μM) resulted in a concentration-dependent inhibition of [^{32}P]PtdOH formation induced by all 3 agonists (fig.1). PDBu (200 nM) exerted effects similar to PMA (fig.2).

Using quin2-labelled platelets, suspended in medium of $[Ca^{2+}]_o$ = 1 mM, PAF (1–100 nM),

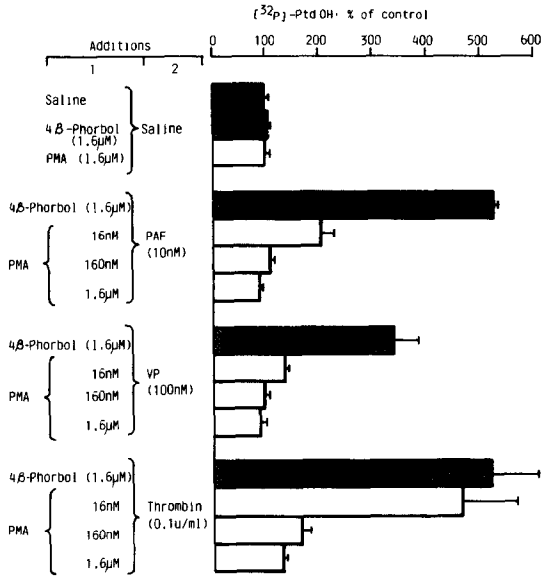

Fig.1. Effects of PMA and 4β-phorbol on PAF-, vasopressin- and thrombin-induced [^{32}P]PtdOH formation in human platelets. Platelets pre-labelled with $^{32}PO_4$ were incubated (2 min, 37°C) with 4β-phorbol (1.6 μM) or PMA at the concentrations indicated prior to addition of PAF (10 nM), vasopressin (VP, 100 nM) or thrombin (0.1 U/ml) for 30 s. Reactions were quenched and phospholipids extracted, separated and the levels of [^{32}P]PtdOH were monitored. Preliminary studies indicated that the effects of 4β-phorbol (1.6 μM) were not significantly different from those of its vehicle DMSO. To facilitate comparison, results (mean values ± SE of triplicate determinations) are expressed as a percentage of the control (saline) sample, in each experiment. Control values (mean ± SE = 352 ± 34 cpm/mg protein) ranged from 202–608 cpm/mg protein in different experiments. The data shown are representative of at least 2 similar experiments performed using platelets obtained from different donors.

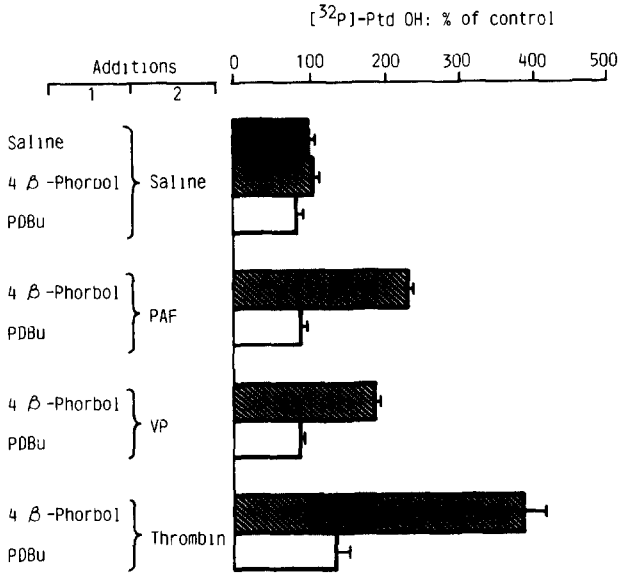

Fig.2. Effects of PDBu on PAF-, vasopressin- and thrombin-induced [^{32}P]PtdOH formation in human platelets. Platelets pre-labelled with $^{32}PO_4$ were incubated (2 min, 37°C) with 4β-phorbol (1.6 μM) or PDBu (200 nM) prior to addition of PAF (10 nM), vasopressin (VP, 100 nM) or thrombin (0.1 U/ml) for 30 s. Other experimental conditions were as stated in the legend to fig.1. The data shown are representative of at least 2 similar experiments obtained using platelets from different donors.

vasopressin (5–500 nM) and thrombin (0.005–0.5 U/ml) cause an elevation of $[Ca^{2+}]_i$ from a resting value of 90 ± 3 nM (mean ± SE, n = 42) to a maximum of 480 ± 100 nM, 420 ± 80 nM and 510 ± 180 nM, respectively (mean ± SE, n = 6–12). When pre-incubated with platelets for 2 min at 37°C, PMA and 4β-phorbol ($\leq$1.6 μM) did not

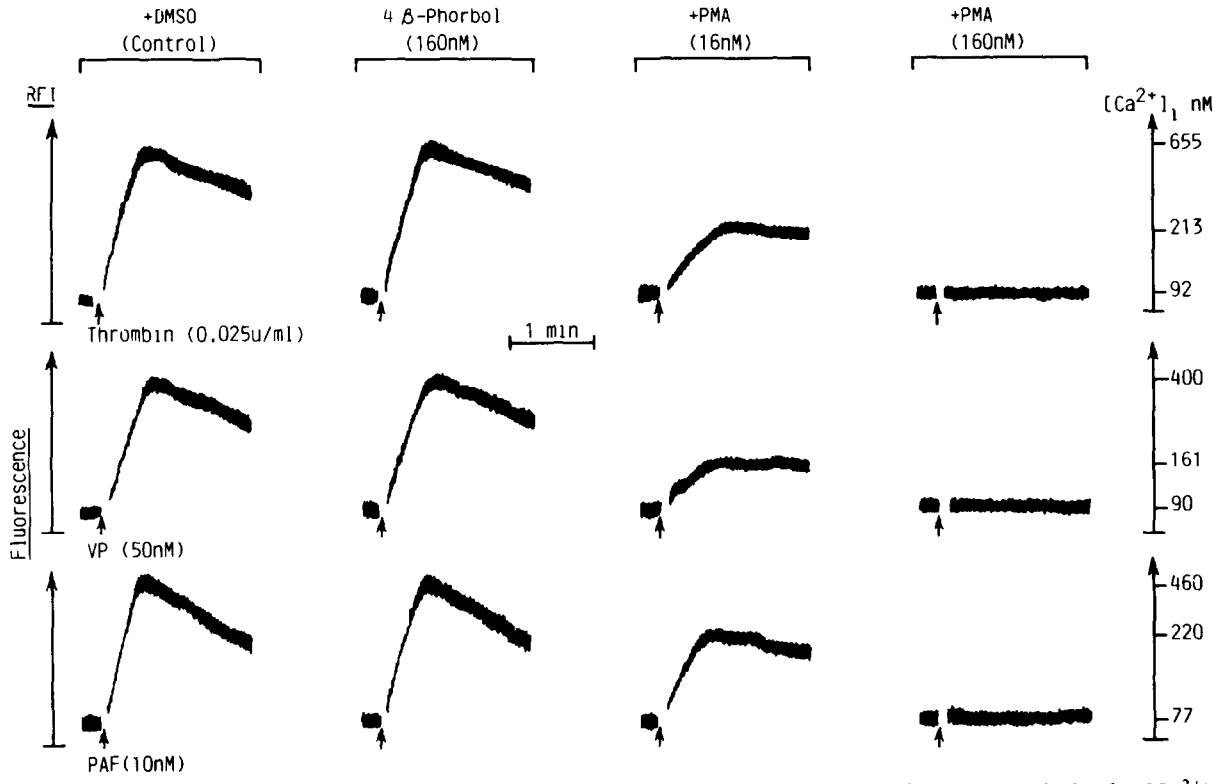

Fig.3. Effects of PMA and 4β-phorbol on PAF-, vasopressin- and thrombin-induced elevation of platelet $[Ca^{2+}]_i$. Plasma-free suspensions of quin2-labelled platelets in a modified Hepes-buffered Tyrodes solution containing 1 mM external Ca^{2+} were incubated (37°C, 2 min) with DMSO (control), 4β-phorbol (160 nM) or PMA at the concentrations indicated. Fluorescence responses (excitation 339 nm, emission 492 nm) of 2 ml samples were monitored following the addition of PAF (10 nM), vasopressin (50 nM) or thrombin (0.025 U/ml). The appropriate intracellular free Ca^{2+} calibration scale is shown at the right of the fluorescence records. The data shown are typical of 3 similar experiments using platelets from different donors.

alter resting $[Ca^{2+}]_i$. However, PMA (16 nM–1.6 μM) but not 4β-phorbol ($\leqslant$1.6 μM) inhibited, in a concentration-dependent manner, the elevation of $[Ca^{2+}]_i$ induced by all 3 agonists (fig.3). PDBu also impaired agonist-induced elevation of platelet $[Ca^{2+}]_i$ (not shown).

4. DISCUSSION

The platelet stimulatory effects of PMA and related tumour-promoting phorbol esters were first recognised 10 years ago [15]. The mechanism(s) underlying such platelet stimulatory effects remained elusive until the demonstration by Nishizuka and colleagues [2,7] that these compounds mimic the effect of endogenous (or exogenous) DAG in activation of protein kinase C. PMA- or DAG-induced platelet functional responses are not accompanied by elevation of platelet $[Ca^{2+}]_i$, thus Rink and colleagues [16] proposed that platelet activation can occur by processes both dependent and independent of elevation of $[Ca^{2+}]_i$. As phosphoinositide hydrolysis may be the receptor-operated molecular event that leads to DAG formation and elevation of $[Ca^{2+}]_i$, it is now believed that agonist-induced platelet activation results, partly at least, from the synergistic interaction of DAG (via activation of protein kinase C) and increased $[Ca^{2+}]_i$ (via activation of Ca^{2+}-calmodulin dependent protein kinases) [4,16].

Such a synergistic interaction between $[Ca^{2+}]_i$ and DAG apparently operates in many systems including adrenal medullary cells, pancreatic islets and neutrophils [17–19]. Besides interacting synergistically with $[Ca^{2+}]_i$ to mediate cellular activation, PMA and related phorbol ester activators of protein kinase C may modulate $[Ca^{2+}]_i$ and influence phosphoinositide metabolism [8,9]. For example, in neutrophils, tumour-promoting phorbol esters have been reported to stimulate arachidonate incorporation into PtdIns [20], directly elevate $[Ca^{2+}]_i$ [8], have no effect on $[Ca^{2+}]_i$ [21], impair agonist-induced elevation of $[Ca^{2+}]_i$ [9] and promote Ca^{2+} extrusion [22,23].

To investigate the possibility that protein kinase C may exert inhibitory effects on the transduction processes that link receptor occupancy to cellular response, we compared the effects of active (PMA, PDBu) and inactive (4β-phorbol) protein kinase C stimulants on phosphoinositide metabolism and Ca^{2+} flux in human platelets. PMA does not alter the resting level of $[Ca^{2+}]_i$ or $[^{32}P]$PtdOH, but suppresses the elevation of $[Ca^{2+}]_i$ and $[^{32}P]$PtdOH formation induced by thrombin, vasopressin and PAF. These effects of PMA are shared by PDBu but not by 4β-phorbol, and thus can be attributed to protein kinase C activation. Suppression by PMA of agonist-induced elevation of $[Ca^{2+}]_i$ could be attributed to activation of a Ca^{2+} extrusion process, as occurs in neutrophils [23]. Were such a mechanism to operate in platelets one might expect to observe a reduction in basal $[Ca^{2+}]_i$. The fact that this was not observed may indicate either that phorbol esters do not activate Ca^{2+} extrusion in platelets, or that Ca^{2+} extrusion is not activated unless there is a concomitant elevation of $[Ca^{2+}]_i$. As phosphoinositide metabolism is implicated in Ca^{2+} mobilisation, there may be a cause and effect relationship between suppression of $[^{32}P]$PtdOH formation and attenuation of Ca^{2+} flux, especially if the former were to reflect inhibition of phospholipase C. However, inhibition of $[^{32}P]$PtdOH formation could be explained by inhibition of PtdIns4P kinase causing depletion of PtdIns4,5P_2 [24], by inhibition of DAG kinase or by activation of PtdOH-phosphohydrolase. Depletion of PtdIns4,5P_2 is unlikely to explain the observed effects, as PMA promotes incorporation of ^{32}P into PtdIns4P > PtdIns4,5P_2 [25], an observation we have confirmed (not shown).

The inhibition of agonist-induced $[^{32}P]$PtdOH formation by PMA and PDBu, reported in this study, is at variance with the findings of Lapetina [26], who observed no inhibition of thrombin (0.5 U/ml)-induced $[^{32}P]$PtdOH formation following incubation of platelets with PDBu (200 nM, 30 s). The reasons for this discrepancy are unknown but may be related to the differences in incubation conditions or agonist concentration utilised in the two studies.

Although the effects of tumour-promoting phorbol esters on platelet phosphoinositide metabolism await clarification, the results of this study suggest that protein kinase C may function as a bi-directional regulator of platelet function. It can act independently, or synergistically with $[Ca^{2+}]_i$, to mediate platelet activation, and it can suppress the transduction processes that link receptor occupancy to cellular response. These divergent

effects may be attributed to the degree of protein kinase C activation or expression. Clearly agonist-induced platelet activation, associated with phosphoinositide hydrolysis, and elevation of $[Ca^{2+}]_i$ occur under conditions where expression of protein kinase C is sub-maximal. Inhibition of receptor-mediated Ca^{2+} flux and [^{32}P]PtdOH formation may require a greater degree of protein kinase C activation or expression: this may be achieved either by prior exposure of platelets to tumour-promoting phorbol esters or following prolonged exposure of platelets to agonists. The latter may be of importance in the termination of the transduction processes involved in platelet activation and may be implicated in the onset of homologous or heterologous desensitisation of platelet responsiveness. Whether such mechanisms operate in other cell types remains to be determined.

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