12-*O*-Tetradecanoylphorbol 13-acetate stimulates inositol lipid phosphorylation in intact human platelets

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The phorbol esters are among the most potent tumor promoters. On addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to isolated human platelets prelabelled with [^{32}P]orthophosphate we found a rapid increase in ^{32}P incorporation into phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. In view of similar findings with cells infected with the oncogene Rous sarcoma virus, it is suggested that inositol lipid phosphorylation might be a key event in the molecular action of phorbol esters.

Polyphosphoinositide *Phorbol ester* *Protein kinase C*

1. INTRODUCTION

The phorbol esters are among the most potent tumor promoters thus far described (review [1]). Although they have a variety of effects on intact cells and membranes, their exact mode of action is still poorly understood [1,2]. The primary effect of these agents appears to occur on the cell membrane; a pleiotropic effect on growth and differentiation most likely represents secondary effects triggered by their initial binding to membrane-associated receptors [3,4].

Authors in [5,6] recently showed that tumor-promoting phorbol esters activated protein kinase C and that the enzyme itself might be a receptor.

Protein kinase C was discovered by the same group and identified as a Ca^{2+}- and phospholipid-dependent enzyme (review [7–9]). In intact cells, it is activated by DAG, generated when various Ca^{2+}-linked receptors induce breakdown of inositol phospholipids by activation of phospholipase C [9–14]. The C kinase presumably plays an analogous role to that of protein kinase A in signal-transducing systems that use cAMP as second messenger.

In recent years the polyphosphoinositide PIPP came into focus as the primary substrate for the receptor-coupled phosphodiesteratic attack (review [15]). The reaction products, DAG and inositol triphosphate are suggested to act as second messengers, respectively, in activating the C kinase [9,16,17] and in releasing Ca^{2+} from intracellular stores [18,19].

A possible connection between oncogene action and polyphosphoinositide metabolism was provided by authors in [20]. They found that the purified Rous sarcoma virus transforming gene product $pp60^{v-src}$ phosphorylated PI to PIP and PIPP. Rous sarcoma virus infected cells showed increased biosynthetic labelling of PIP and PIPP. The authors suggested that, through the increase in polyphosphoinositides, an amplified generation of second messengers might activate pathways leading to the malignant phenotype; one of the key events would be the activation of the C kinase.

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DAG, diacylglycerol; PIPP, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; OAG, 1-oleoyl-2-acetylglycerol; OAPA, 1-oleoyl-2-acetyl-3-phosphoric acid

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To assess a correlation between the activation of the C kinase by phorbol esters and the possible involvement of polyphosphoinositides in malignant transformation we studied the influence of TPA on polyphosphoinositide metabolism.

2. EXPERIMENTAL

Human venous blood was collected from healthy volunteers in 0.2 vol. anticoagulant buffer containing 1.3% citric acid, 2.5% sodium acetate and 2% dextrose.

Platelets were prepared as in [21], except for the buffers. Washing buffer contained 25 mM Hepes (pH 7.2), 125 mM NaCl, 10 mM glucose, 1 mM EGTA and 1 mg/ml BSA. Final buffer consisted of 25 mM Hepes (pH 7.5), 125 mM NaCl, 2.7 mM KCl, 5.6 mM $MgSO_4$, 10 mM glucose, 0.1 mM EGTA, 1 mg/ml BSA. After addition of carrier-free [^{32}P]orthophosphate (250 μCi/ml platelets), the platelet suspension was incubated at 37°C for 60 min. TPA (Sigma) or OAG were added in DMSO or ethanol (1‰ final solvent concentration). Samples (150 μl: 4×10^8 cells) were taken at the time indicated and immediately transferred in 4.5 ml $CHCl_3/CH_3OH$/0.45 N HCl (20:40:13). Two phases were obtained after the addition of 1.25 ml $CHCl_3$ and 1.25 ml of 0.1 N HCl. After centrifugation the organic phase was washed with 2.5 ml CH_3OH/0.2 N HCl (1:1) and recentrifuged. The organic phase was evaporated under N_2, applied to silica gel 60 precoated plastic sheets (Merck, FRG) and eluted in $CHCl_3/CH_3OH$/20% methylamine (60:35:10). After autoradiography on Kodak X-Omat R films, phospholipids were located on the sheets and cut out. Radioactivity was quantitated by liquid scintillation spectrometry.

For protein analysis, 80-μl samples (2.1×10^8 cells) were quenched in a 5-fold concentrated sample buffer [22] and incubated at 90°C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12.5% gel using the buffers in [22]. Gels were stained with Coomassie brilliant blue, dried and subjected to autoradiography. Areas corresponding to the 40-kDa and 20-kDa proteins were cut out and radioactivity was determined by liquid scintillation counting.

3. RESULTS

In our experiments we compared the changes in ^{32}P-labelled phospholipids with changes in protein kinase C activity. Isolated human platelets were prelabelled with [^{32}P]orthophosphate for 1 h. At that time cellular ATP, PIP and PIPP pools reached isotopic equilibrium and therefore changes in ^{32}P content would reflect changes in mass. Protein kinase C activity in the intact platelet can easily be quantified by the incorporation of ^{32}P label in its major substrate, a 40-kDa protein.

On addition of TPA to isolated human platelets prelabelled with [^{32}P]orthophosphate, the amount of label in PIP increases to about 300% of the controls within 2 min (fig.1). Changes in [^{32}P]PIPP became significantly ($p < 0.05$) different from control values after 1 min. The phosphorylation of 40-kDa protein reaches 90% of its maximum within 30 s after addition of phorbol ester (as shown in [5]).

The dose dependency of [^{32}P] incorporation in the polyphosphoinositides and 40-kDa protein is il-

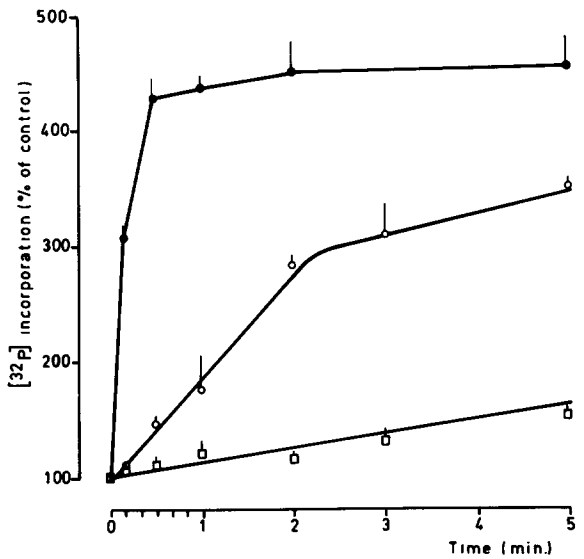


Fig.1. Time-dependent changes in ^{32}P-labelled 40-kDa protein, [^{32}P]PIP and [^{32}P]PIPP on addition of TPA (10 ng/ml) to isolated human platelets prelabelled with [^{32}P]orthophosphate. (●) ^{32}P-labelled 40-kDa protein (average for 100%, 404 cpm), (○) [^{32}P]PIP (average for 100%, 14211 cpm), (□) [^{32}P]PIPP (average for 100%, 21202 cpm). The points represent means ± SD of 4 experiments with duplicate samples.

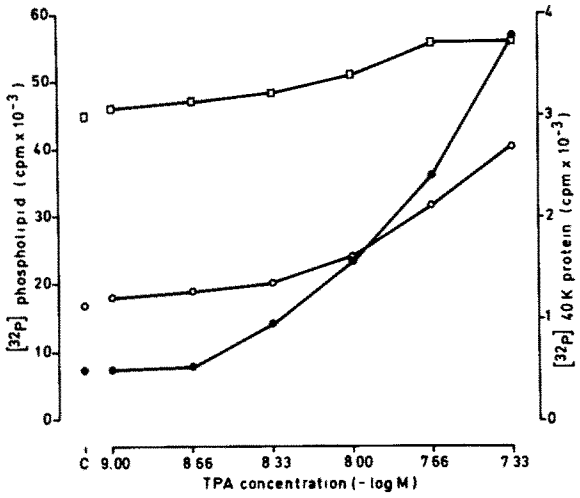


Fig.2. Changes in ^{32}P-labelled 40-kDa protein, [^{32}P]PIP and [^{32}P]PIPP on addition of different concentrations of TPA to isolated human platelets prelabelled with [^{32}P]orthophosphate. Protein and lipid samples were taken at 60 and 70 s after the addition of the phorbol ester, respectively. (●) ^{32}P-labelled 40-kDa protein, (○) [^{32}P]PIP, (□) [^{32}P]PIPP. The points represent mean values of duplicate sample. Results are representative for 3 separate experiments.

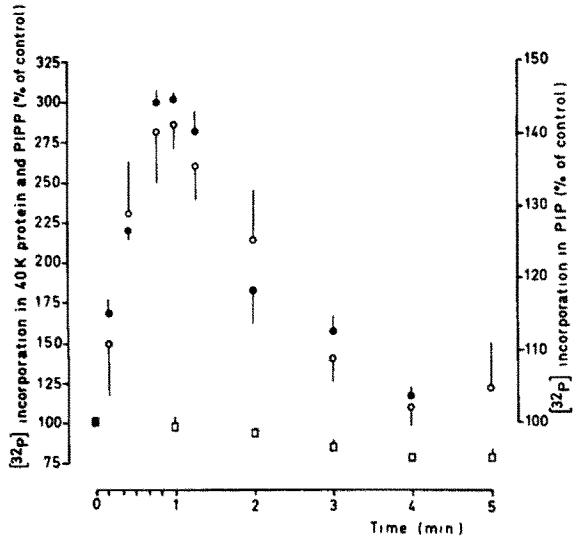


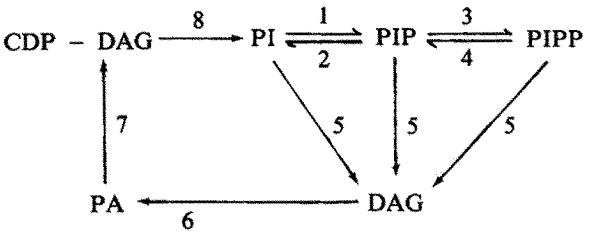
Fig.3. Time-dependent changes in ^{32}P-labelled 40-kDa protein, [^{32}P]PIP and [^{32}P]PIPP on addition of OAG (5×10^{-6} M) to isolated human platelets prelabelled with [^{32}P]orthophosphate. (●) ^{32}P-labelled 40-kDa protein (mean ± SD of 5 experiments with duplicate samples and 1 experiment with quadruplicate samples; average for 100%, 467 cpm). (○) [^{32}P]PIP (mean of 6 experiments with duplicate samples; average for 100%, 18523 cpm). (□) [^{32}P]PIPP (mean of 6 experiments with duplicate samples; average for 100%, 33272 cpm).

lustrated in fig.2. In absolute terms of ^{32}P incorporated, major changes are observed in PIP while the percentage increase is highest for 40-kDa protein phosphorylation. ^{32}P incorporation in PI and PC did not change significantly during 5 min contact time with the phorbol ester (not shown). [^{32}P]PA always decreased slightly but the differences from control became significant ($p < 0.05$) only after 5 min (not shown).

To illustrate a possible correlation between the activation of the C kinase and the phosphorylation of inositol lipids the influence of the synthetic DAG, OAG, on prelabelled platelets is illustrated in fig.3. OAG intercalates in the cell membrane and directly activates the C kinase without involvement of receptor generated DAG [23]. At the concentration of OAG used (5×10^{-6} M), ^{32}P-labelled 40-kDa protein phosphorylation is transient (fig.3). Apparently the lipid is rapidly converted into OAPA with subsequent loss of activation of the kinase; this is in contrast to TPA that is not readily metabolized and activates the kinase persistently. The incorporation of ^{32}P into PIP completely parallels ^{32}P-labelled 40-kDa protein formation. The amount of [^{32}P]PIPP decreases (fig.3).

4. DISCUSSION

The increase in ^{32}P-labelled polyphosphoinositides, as observed on addition of TPA to platelets, might be ascribed to their decreased breakdown and/or increased synthesis. The possibility of an inhibition of phospholipase C activity (scheme 1) seems rather unlikely because of its low basal levels [24]. Any further inhibition would hardly produce the drastic increases we found (fig.2). Therefore, changes in the activity of enzymes involved in the synthesis and dephosphorylation of the inositol lipids (scheme) most probably account for our observations. Since the levels of [^{32}P]PIP and [^{32}P]PIPP both change, it is impossible, from our results, to locate the altered enzymatic activity(ies). However, it should be mentioned that the increase in label we found tends to reflect changes in mass since both polyphosphoinositides are labelled at



Scheme 1. Pathways for synthesis and breakdown inositol phospholipids. 1, PI kinase; 2, PIP phosphomonoesterase; 3, PIP kinase; 4, PIPP phosphomonoesterase; 5, phospholipase C; 6, DAG kinase; 7, CTP-phosphate cytidyltransferase; 8, CDP:1,2-DAG inositol phosphatidyltransferase. Note: the inositol exchange enzyme is not included since it cannot account for changes we measure in our experiments.

near isotopic equilibrium, so that the results are suggestive of an increase in lipid kinase activity.

The $pp60^{v-src}$, the transforming gene product of Rous sarcoma virus that is supposed to be involved in the oncogene activity in transformed cells, was recently found to catalyse also PIP and PIPP formation [20]. It is intriguing that addition of TPA, a tumor promoter, also induces a marked increase in polyphosphoinositide formation. If the latter is important in carcinogenesis, it becomes evident how this primary effect of TPA on intact cells might contribute to tumor promotion. However, the physiological implication of the polyphosphoinositide synthesis is still highly speculative. The suggested potential increase in second messenger [20], although very attractive, remains to be elucidated.

TPA is known to stimulate the C kinase [5,6,25]. To evaluate the possible metabolic relationship between the activation of the protein kinase and polyphosphoinositide formation, the effect of OAG is illustrated in fig.3. This synthetic DAG activates the C kinase directly [23] without the involvement of receptor-mediated DAG generation, thus acting similarly to TPA [5,6]. The correlation between ^{32}P-labelled 40-kDa protein and [^{32}P]PIP formation is evident: both are transient (due to the enzymatic conversion of OAG to OAPA) and follow the same time course. However, in contrast to our findings on addition of TPA, [^{32}P]PIPP decreases with OAG. An explanation for this difference is as yet not evident and must await further insight into the exact working mechanism of both products. From our results, however, it is clear that TPA and OAG, that are both known to activate the C kinase directly, do also prime polyphosphoinositide formation. The permanent nature of the stimulation found with the phorbol ester compared to the transient one of OAG (and most likely any receptor coupled endogenously formed DAG) stresses its potential importance for the long-term action of this tumor promoter in carcinogenesis.

TPA has been reported to alter lipid metabolism in intact cells. It stimulates the breakdown as well as the resynthesis of PC [26–28]. DAG is also reported to accumulate on addition of TPA to myoblasts [29] and A431 cells [30]. Phospholipase C activity on endogenous phospholipids would be the causal factor. None of these observations can be regarded as a primary response since they occur at a much later stage after addition of TPA than the changes in phospholipid metabolism we observed.

In conclusion, evidence is presented that TPA stimulated polyphosphoinositide formation immediately after its addition to human platelets. Since the primary actions of phorbol esters are supposed to occur at the cell membrane, the triggering of this early change in phospholipid composition might be a key event in the molecular action of the phorbol esters.

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