Phorbol ester and 1,2-diolein are not fully equivalent activators of protein kinase C in respect to phosphorylation of membrane proteins in vitro

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Phosphorylation of liver plasma membrane proteins by protein kinase C was studied by using the two best known activators of the enzyme, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1,2-diolein. While the effects of TPA and diolein were almost identical on two proteins and similar in magnitude on four proteins, the phosphorylation of an additional four proteins was increased only by TPA. We conclude that in respect to phosphorylation of membrane proteins, TPA and diglycerides are not fully equivalent activators of kinase C.

12-O-Tetradecanoylphorbol 13-acetate *1,2-Diolein* *Protein kinase C*

1. INTRODUCTION

The phospholipid- and calcium-dependent protein kinase (kinase C) can be activated by both 1,2-DG and the tumor-promoting phorbol esters [1,2]. In fact, the enzyme appears to be identical with the phorbol ester receptor [3–6]. The physiological activator of the enzyme is 1,2-DG which accumulates transiently as a consequence of increased inositol lipid breakdown [7,8]. Kinase C is expected to be an intracellular mediator for all hormones which stimulate the hydrolysis of inositol lipids [9].

A commonly held view is that phorbol esters and 1,2-DG should act in a similar manner and, indeed, many cellular events are similarly induced by these agents [10–16]. Although 1,2-DG often has smaller effects than phorbol esters, this has usually been attributed to the relatively rapid metabolism of 1,2-DG in intact cells. Most recent reports, however, suggest that the inability of 1,2-DG to reproduce the effects of phorbol esters on differentiation of HL60 cells [17], maturation of granulosa cells [18] and platelet activation [19] is not due to its rapid metabolism. This would indicate that 1,2-DG has a somewhat narrower range of action in vivo compared to phorbol esters.

There are at least two possible mechanisms which could account for the differences between the effects of 1,2-DG and phorbol esters: (i) kinase C, as proposed in [18], may not be the sole target of phorbol esters; (ii) activation of kinase C by either phorbol esters or 1,2-DG does not result in identical phosphorylation patterns. The latter phenomenon was observed by one of us in intact S49 mouse lymphoma cells [20].

Here, we compared the effect of TPA, a potent tumor-promoting phorbol ester, and 1,2-diolein on the kinase C-mediated phosphorylation of liver

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Abbreviations: PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 1,2-DG, 1,2-diacylglycerol

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plasma membrane proteins. The advantage of using this system is that while it contains kinase C [21] and many of its substrates, one can avoid certain complicating features of the intact cells such as the possible compartmentalization and metabolism of kinase C activators and the enzyme itself. We will show qualitative differences in the stimulatory effects of TPA and diolein on the phosphorylation of membrane proteins.

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphatidylserine, 1,2-diolein, TPA and histone II-S were from Sigma. Acrylamide, SDS, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were bought from Serva. Ampholines (pH range 3.5–10 and 5–7) were the product of LKB. [γ-^{32}P]ATP (~1000 Ci/mmol) was prepared by the Isotope Institute of the Biological Research Center (Szeged). X-ray film (Medifort RP) and photographic chemicals were from Forte Photochemical Industry (Vac, Hungary) and intensifying screens (Lightning plus) from Du Pont.

2.2. *Methods*

Female Wistar rats (150–180 g body wt) that had been starved overnight were used. Liver plasma membranes were prepared according to Neville [22].

2.2.1. ^{32}P labelling of membrane proteins

Protein kinase C was solubilized from plasma membranes by using 1 mM EGTA in a volume of 1 ml containing also 25 mM Tris-HCl (pH 7.6) and 3 mg membrane proteins. Solubilization was performed at 4°C for 1 h by occasionally stirring the samples. To measure endogenous protein phosphorylation, 30-μl aliquots of the above mixture were added to the assay mixture (0.1 ml final volume) which contained 25 μM [γ-^{32}P]ATP (20000 cpm/pmol), 10 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.6), 20 μg PS and 2 μg 1,2-diolein (or 50 nM TPA). Incubations were performed for 5 min after the addition of membranes. Due to the relatively rapid hydrolysis of ATP by membrane-bound ATPases the concentration of ATP dropped below 5 μM by 2.5 min incubation. Therefore, at 2.5 min we added labelled ATP again (in 5 μl) to restore the original ATP concentration (both labelled and unlabelled). There was no further increase in protein phosphorylation after 5 min even if ATP was repeatedly added. In fact, most proteins were maximally phosphorylated after 1 min incubation. Incubations were stopped and membrane proteins prepared for gel electrophoresis as in [23].

2.2.2. Two-dimensional polyacrylamide gel electrophoresis

The two-dimensional gel procedure of O'Farrell [24] was used with the modifications described by Steinberg and Coffino [25]. For all samples of a single experiment, first-dimension isoelectric focusing gels were loaded with equal amounts of proteins (90 μg): the acid-precipitable radioactivity in any of the samples varied by less than 10%. Gels were run for 7000 V·h; second-dimension SDS-containing gels were 10% (w/v) in polyacrylamide. Labelled proteins were detected by autoradiography of dried gels for 2–3 days at −70°C using lightning plus intensifying screens. Gel patterns are shown with the acidic proteins to the right. The appropriate phosphoproteins were cut out and their radioactivity directly measured in a dioxane-based scintillation cocktail using an LKB liquid scintillation spectrometer. Protein was determined according to Lowry et al. [26].

3. RESULTS

Rat liver plasma membranes, purified by Neville's procedure, contain kinase C activity [21]. The membrane-bound enzyme, however, cannot be induced by PS plus diolein [21] or PS plus TPA (unpublished) to phosphorylate exogenous substrates. As documented in fig.1c, addition of PS plus TPA to plasma membranes also failed to increase phosphorylation of endogenous proteins. Activation of membrane-bound kinase C by lipids and TPA usually requires the release of the enzyme by either EGTA [21,27,28] or detergents [29–31]. Thus, we first pretreated the membranes with EGTA and then studied the phosphorylation of endogenous proteins by the solubilized kinase C. During the phosphorylation assay the original ratio of kinase C to membrane proteins was maintained. As shown in fig.1b, EGTA treatment alone decreased the radioactivity in several spots

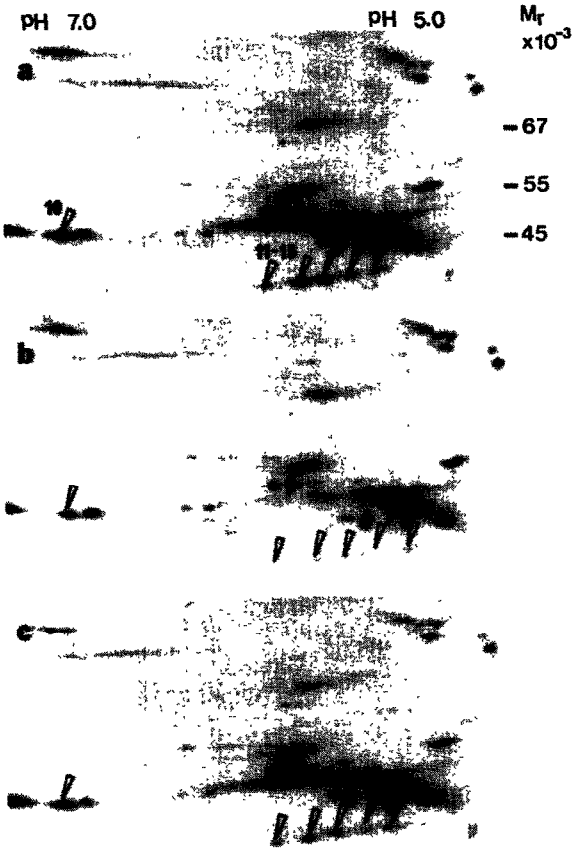

Fig.1. Effect of EGTA and PS plus TPA on the phosphorylation of liver plasma membrane proteins. For ^{32}P labelling of proteins, membranes were used without pretreatment (a) or after pretreatment with 1 mM EGTA for 1 h at 4°C (b). In (c) 20 μg PS plus 50 nM TPA were added to untreated membranes. From each sample portions (90 μg protein) containing about 10^5 cpm acid-precipitable radioactivity were subjected to high-resolution two-dimensional gel electrophoresis: gels were dried and exposed for 2 days at −70°C using lighting plus intensifying screens. Open arrowheads indicate those proteins whose phosphorylation was inhibited by EGTA.

(designated as proteins 10–15). Most probably they are not related to the removal of kinase C, since when EGTA treatment was followed by centrifugation (and the solubilized kinase C was discarded) addition of 0.1 mM $CaCl_2$ to membranes alone restored the levels of phosphorylation (not shown).

Addition of optimum concentrations of PS (0.2 mg/ml) plus TPA (50 nM) to the mixture of membranes and solubilized kinase C resulted in increased phosphorylation of 10 proteins (fig.2b). When applied alone, TPA had smaller but still significant effects while PS had no effect at all (not shown). On the other hand, when optimum amounts of PS plus diolein (20 μg/ml) were added together (fig.2c) phosphorylation of only 6 of the above 10 proteins was increased. Addition of $CaCl_2$, tested up to 0.1 mM, did not alter the effects of TPA and diolein (not shown). Most probably, activation of kinase C by these agents requires such small amounts of calcium which were already present even in the presence of 0.3 mM EGTA (the final concentration of chelator in the assay mixture). This is consistent with the observation of others [32]. No effect of TPA (fig.2d) or diolein (not shown) was observed, however, at 3 mM EGTA. Addition of trifluoperazine at 50 μM, a known inhibitor of kinase C [29], completely blocked the stimulatory effects of both TPA and diolein (not shown).

Table 1

Summary of the effect of TPA and 1,2-diolein on the phosphorylation of plasma membrane proteins

Protein no.	Stimulation by	
	TPA	1,2-Diolein
1	+ + +	−
2	+ +	+ +
3	+ + +	+ + +
4	+ + +	+ + +
5	+ + +	+ + +
6	+ +	−
7	+ +	−
8	+ +	+
9	+ +	+ +
10	+ +	−

+, + + and + + + indicate 1–2-, 2–4- and more than 4-fold increases in protein phosphorylation beyond basal level (observed in the absence of kinase C activators). Data are based on direct measurement of ^{32}P radioactivity in proteins. For this purpose, 3 gels, derived from 3 different experiments, were used

The effects of TPA and diolein on the phosphorylation of membrane proteins, derived from the experiment shown in fig.2 and two other experiments, are summarized in table 1. In the case of proteins 3 and 9 the stimulatory effects of

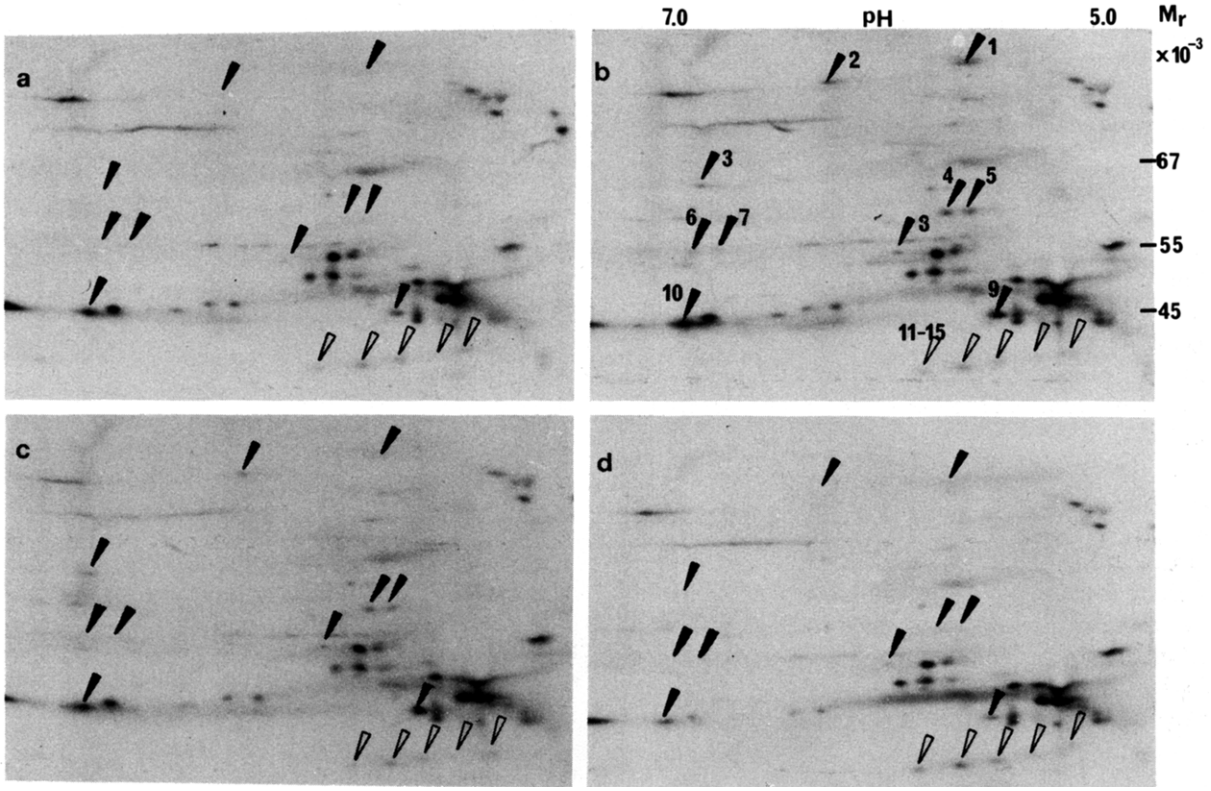

Fig.2. Effect of PS, TPA, 1,2-diolein and high concentration of EGTA on the phosphorylation of membrane proteins after treatment with EGTA. After treatment with 1 mM EGTA for 1 h at 4°C, membranes were used for ^{32}P labelling of proteins with no additions (a), in the presence of PS plus TPA (b), PS plus 1,2-diolein (c) or PS plus TPA plus 3 mM EGTA (d). Concentrations of PS, 1,2-diolein and TPA were 20 μg/assay, 2 μg/assay and 50 nM, respectively. Note that in a–c, the final concentration of EGTA was 0.3 mM. Samples (90 μg protein and about 7×10^4 acid-precipitable cpm) were subjected to gel electrophoresis as before and dried gels exposed for autoradiography for 2 days at −70°C using lighting plus intensifying screens. Open and filled arrowheads indicate those proteins whose phosphorylation was either decreased by EGTA or increased by PS plus TPA or PS plus diolein.

diolein and TPA were of similar extent while diolein, compared to TPA, had smaller but still clear effects on proteins 2, 4, 5 and 8. Diolein did not significantly increase the phosphorylation of proteins 1, 6, 7 and 10 during the 5 min incubation period. The discrepancy between the effects of TPA and diolein is particularly evident in the case of proteins 1 and 10 whose phosphorylation is increased by TPA by 5- and 3-fold, respectively.

4. DISCUSSION

The present experiments demonstrate increased phosphorylation of 10 liver plasma membrane proteins by solubilized kinase C in the presence of PS and TPA. Of these proteins 6 showed increased phosphorylation in the presence of diolein while this lipid had no significant effect on the 4 other proteins. These results extend our previous observations on intact S49 lymphoma cells [20] that stimulation of kinase C with TPA and diglycerides does not result in the same phosphorylation pattern. The significance of this observation may be the following. Kinase C molecules, which are present in the cytoplasm in varying proportions [28], are rapidly transferred to plasma membranes when the cells are treated with TPA or agents which elevate the internal level of diglycerides [33–36]. A major site of kinase C action thus appears to be the plasma membrane. One would expect that if TPA and diglycerides did not induce identical phosphorylation patterns in the plasma membrane

then there would also have been differences in their physiological actions. Indeed, recent evidence indicates differences in the mode of actions of TPA and diglycerides in vivo [17–19].

The reason for the discrepancy between the effects of TPA and diolein on kinase C-mediated endogenous phosphorylation is not well understood at present. However, it may be important to recall that while one part of the TPA and diglyceride molecules is similar (that which is probably recognized by the enzyme), the other is strikingly different (which is probably involved in interaction with membranes). Due to this difference, kinase C molecules in the presence of TPA or diolein may: (i) bind to different sites in the membrane, (ii) have different mobilities in the membranes, or (iii) have somewhat different substrate specificity. While further studies are required to distinguish among these possibilities it is already clear that one does not necessarily have to postulate additional mechanisms for TPA actions (not involving kinase C) in order to explain the somewhat narrower range of diglyceride action.

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