# Separation from protein kinase C – a calcium-independent TPA-activated phosphorylating system

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A calcium-independent but 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- or diacylglycerol-activated phospholipid-dependent phosphorylating activity has been separated from protein kinase C. This has been made possible by employing calcium-dependent hydrophobic interaction chromatography. The material bound to phenyl-Sepharose in the presence of calcium at low ionic strength was eluted with EGTA and was protein kinase C. While the unbound material passing through the phenyl-Sepharose column showed no appreciable protein kinase C activity, instead it had a high phosphorylating activity manifested in the absence of calcium and in the presence of TPA plus phospholipid. The identification of this phosphorylating activity, distinct from protein kinase C, leads to important clues to cellular responses monitored by TPA in the absence of calcium.

*Protein kinase C* *Phorbol ester* *Tumor promotion* $Ca^{2+}$

## 1. INTRODUCTION

It is taken for granted that the cellular responses triggered by the tumor promoter, TPA, involve calcium-phospholipid dependent protein kinase (protein kinase C) [1,2]. Such is the current trend towards the understanding of the molecular basis of tumor promotion [3]. One of the intriguing aspects of TPA action is the observed lowering of the calcium requirement in manifesting protein kinase C activity [4]. In an attempt to address this question the physical separation of phosphorylating activity which is entirely dependent on phorbol ester plus phospholipid, but independent of calcium, is demonstrated here. The evidence is provided in this report that protein kinase C is activated by a natural DAG containing two long fatty acid chains, thus mimicking phorbol ester. The identification of a kinase system unconnected with calcium but still dependent on phorbol ester and phospholipid may give insight into several cellular responses triggered by TPA in the absence of calcium [5–7].

*Abbreviations:* PS, phosphatidylserine; PI, phosphatidylinositol; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

## 2. MATERIALS AND METHODS

Rat brain was homogenized in a buffer containing 20 mM Hepes (pH 7.5), 300 mM sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 10 $\mu$g pepstatin A/ml, 10 $\mu$g leupeptin/ml, 10 $\mu$g aprotinin/ml, 10 $\mu$g soyabean trypsin inhibitor/ml, 10% glycerol and centrifuged at 100000 × *g* for 1 h. Resulting supernatant was applied to a Blue Sepharose CLGB column (previously equilibrated in 20 mM Hepes buffer, pH 7.5, 0.5 mM each of EDTA and EGTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10% glycerol), washed with 0.4 M KCl in the same buffer and eluted at 1.4 M KCl. The active fractions were pooled and dialyzed against the same buffer minus KCl and $MgCl_2$ for 6 h. Protein was precipitated by 70% ammonium sulfate. The sedimented material was dissolved and applied to an Ultrogel AcA34 column. Active fractions were

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pooled and dialyzed overnight against 20 mM Hepes buffer (pH 7.5) containing 1 mM dithiothreitol, 0.5 mM each of EDTA and EGTA, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 10% glycerol. The dialysate was applied to phenyl-Sepharose column equilibrated with the same buffer. Fractions flowing through this column were tested for their phosphorylating activity and active fractions were pooled. After washing, material bound to phenyl-Sepharose was eluted with 20 mM Hepes buffer (pH 7.5), 0.5 mM EGTA and 2 mM glycerol. The fractions containing protein kinase C activity were pooled. Each one of these fractions (i.e. those passing through and eluted) was separately subjected to DE52 cellulose. After washing with 30 mM NaCl (in 20 mM Hepes buffer (pH 7.5), 0.5 mM each of EDTA and EGTA, 1 mM dithiothreitol, 10% glycerol), the enzyme was eluted with 120 mM NaCl in the same buffer.

DAG was prepared by incubating egg lecithin with phospholipase C in the presence of calcium at 37°C for 2 h as described by Hanahan et al. [8]. Purification of DAG was in two steps: first, on Silic AR (Mallinckrodt) column chromatography eluted with an ether-hexane mixture. Second, the eluent was subjected to thin-layer chromatography on a silica gel 60 plate with benzene/ether/ ethyl acetate/acetic acid (80:10:10:0.2). The DAG spot was eluted, stored in chloroform and quantified spectrophotometrically by measuring the amount of ester bonds using cholesterol acetate as standard.

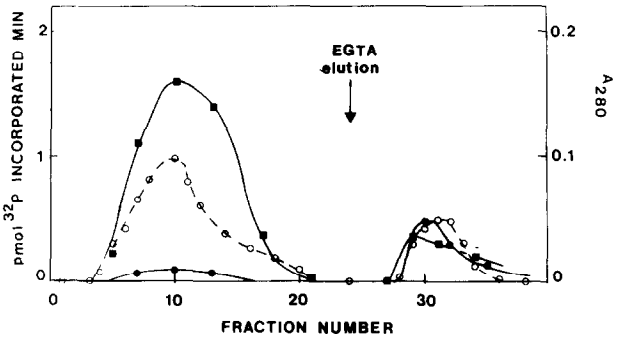


Fig.1. Separation of a phospholipid + TPA phosphorylating activity from protein kinase C employing calcium-dependent phenyl-Sepharose chromatography. Phosphorylation assay in the presence of $Ca^{2+}$ + PS (●), in the presence of PS + TPA (■) and the protein ($A_{280}$) is depicted (○---○). The details of kinase assay were the same as in table 1. 20 μl each of EGTA eluted material was taken as an enzyme source for assay, while the passing through material was 40 μl per assay (kinase-L).

Table 1

Effect of $Ca^{2+}$ on protein kinase specific activities under the influence of various effectors

| | Protein kinase-L (nmol $^{32}$P/min per mg protein) | | Protein kinase C (nmol $^{32}$P/min per mg protein) | |
|---|---|---|---|---|
| | − $Ca^{2+}$ | + $Ca^{2+}$ | − $Ca^{2+}$ | + $Ca^{2+}$ |
| PS | 0.04±0.03 | 0.21±0.12 | 0.09±0.03 | 19.8±2.1 |
| PS + TPA | 5.9 ±0.7 | 6.2 ±0.8 | 23.9 ±2.2 | 24.3±2.2 |
| PS + DAG | 4.3 ±0.9 | 5.2 ±1.1 | 28.8 ±3.0 | 28.9±1.9 |
| PI | 0.04±0.04 | 0.25±0.12 | 0 | 1.6±0.2 |
| PI + TPA | 4.9 ±0.5 | 4.8 ±0.6 | 5.0 ±0.6 | 10.4±1.2 |
| PI + DAG | 4.6 ±1.1 | 4.8 ±0.8 | 1.9 ±0.2 | 8.8±0.9 |

Standard protein kinase assay was essentially the same as in [16]. However, the concentration of additions was: 1.75 mM $Ca^{2+}$, 20 μM ATP (3-5 × $10^5$ cpm), 16 μg/ml PS or PI, 1.2 mM EGTA, 50 ng/ml TPA, 100 μM DAG and 2 mM phenylmethylsulfonyl fluoride. Incubations were carried out at 30°C for 15 min. Protein was estimated according to Bradford [17]. Results are expressed as mean ± SD of 3 experiments

## 3. RESULTS

Fig.1 illustrates physical separation from protein kinase C of a phosphorylating activity solely dependent on PS + TPA in the absence of calcium. This activity was observed in the passing through material of the phenyl-Sepharose column. For convenience, this activity is termed kinase-L activity, due to its phospholipid dependency. The material obtained after elution with EGTA was protein kinase C and showed an orthodox calcium + PS phosphorylation response.

Table 1 presents the phosphorylating activity of protein kinase C as contrasted from the activity of the phenyl-Sepharose column passing through material (hitherto termed protein kinase-L). In the first instance the phosphorylating activity in the presence of calcium + PS was very striking in the case of protein kinase C as compared with kinase-L. Secondly, there was a tremendous phosphorylation activity manifested by kinase-L upon TPA or DAG addition in the presence of PS as compared to the activity observed in the presence of PS alone. No significant change in this activity was observed when calcium was added to the assay conditions. Whereas protein kinase C followed calcium-phospholipid dependency, in the presence of PS + TPA (absence of calcium) the phosphorylation level reached almost that of $Ca^{2+}$ + PS. DAG produced a similar effect to that of TPA. No phosphorylation was observed when TPA or DAG were added in the absence of phospholipid (not shown).

The nature of response observed when phosphatidylinositol (PI) was substituted for PS was almost identical so far as kinase-L was concerned. In the case of protein kinase C, less pronounced phosphorylation was seen with $Ca^{2+}$ + PI as contrasted with that observed with $Ca^{2+}$ + PS. The magnitude of increase in protein kinase C activity in the presence of calcium, PI + TPA was larger than the corresponding value elicited in the presence of PS. Natural DAG used here demonstrated analogy with the effect seen with TPA.

## 4. DISCUSSION

A physical separation of phosphorylating activity (kinase-L) solely dependent on phospholipid plus phorbol ester (or DAG) from orthodox calcium-phospholipid dependent protein kinase (protein kinase C) is the main aim of this manuscript. It is largely agreed that phorbol ester lowers the optimal calcium requirement for the manifestation of protein kinase C activation. However, this is the first report demonstrating physical distinction between a phosphorylating activity expressed in the absence of calcium and the classical protein kinase C, although both activities are influenced by phorbol ester or DAG. Such a separation of a calcium-independent system from calcium-dependent kinase C could be invoked by the use of calcium-dependent hydrophobic interaction chromatography. The rationale lies in the fact that protein kinase C binds phenyl-Sepharose in the presence of calcium at low ionic strength [9] and is eluted with EGTA.

The effect of TPA or DAGs on protein kinase C either in the presence of calcium or in its absence follows the orthodox pattern. For example the activity of kinase C in the presence of phospholipid plus TPA is almost identical to the activity observed with calcium + PS. This is in total agreement with the observations of Arcoleo and Weinstein [10]. Furthermore, TPA does not seem to substantially enhance the phosphorylation activity over the level already attained in the presence of calcium + PS in the case of kinase C.

So far as kinase-L is concerned, firstly, there is no appreciable activity observed in the presence of calcium plus phospholipid (PS or PI). Secondly, the addition of TPA or DAG dramatically increases the activity observed in the presence of phospholipid (PS or PI) alone, and it is this activation which remains insensitive to calcium. This distinction becomes more pronounced in the case of PI. With respect to kinase-L, the PI + TPA or PI + DAG response is not influenced when calcium is added to the assay medium. But in the case of kinase C, calcium enhances further the phosphorylation activity attained with PI + TPA or PI + DAG although the calcium + PI response is much lower than the response elicited by calcium + PS.

Based on the above arguments, it is reasonable to postulate that the phosphorylation observed with the kinase-L moiety is distinguishable from protein kinase C and is in fact separated from the latter. The calcium-independent nature of protein kinase-L activated by TPA may provide a

mechanistic basis for certain cellular responses triggered by TPA in the absence of calcium. Some such responses are: (i) TPA stimulation of EGF receptor phosphorylation without calcium [5]; (ii) TPA-induced protein phosphorylation and exocytosis in neutrophils independent of intracellular calcium [6]; (iii) the effect of TPA on lymphocyte proliferation occurring in the absence of extracellular calcium or detectable changes in intracellular calcium level [7].

A word about the effect of TPA and DAG deserves a mention here. TPA is implicated to bear structural resemblance with 1-oleolyl-2-acetyl glycerol [12]. Synthetic DAGs having fatty acid chains up to 10 carbons have also been shown to activate protein kinase C [13,14]. Here evidence is provided that protein kinase C is activated by a natural DAG containing two long fatty acid chains analogous to the TPA effect. The natural DAG is also shown to resemble TPA in activating kinase-L in a calcium-independent manner. This provides additional support that the site of action for DAG and phorbol ester is the same.

Returning to kinase-L as compared to kinase C activity, it may be argued that the two phosphorylation activities share certain common features: (i) histone H1 is their preferred substrate; (ii) they do not phosphorylate H2 or casein (not shown); (iii) phospholipids are required for their activity. This reveals a certain kinship between the two. Is kinase-L a protein kinase C which has lost its calcium binding site and therefore could be separated on calcium-dependent hydrophobic chromatography? Does there exist an equilibrium between two conformations (C and L) of the same enzyme? In this hypothesis it is the kinase-L conformation which is the actual target of TPA or DAG action. These pertinent questions are raised here opening a new avenue to address the role implicated for protein kinase C in tumor promotion. Nevertheless, these data advance the understanding of the molecular basis of TPA activation of protein kinase C at a lower or zero calcium concentration [10,15].

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