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Phosphorylation of an acidic mol. wt. 80 000 cellular protein in a cell-free system and intact Swiss 3T3 cells: a specific marker of protein kinase C activity

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Activation of the endogenous Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C) by Ca^{2+}, phosphatidylserine (PS) and phorbol dibutyrate (PBt_2) in detergent-solubilized extracts of Swiss 3T3 cells resulted in a very rapid increase (detectable within seconds) in the phosphorylation of an 80 000 mol. wt. protein (termed 80 K). Neither cyclic AMP nor Ca^{2+} had any effect on 80 K phosphorylation. The 80 K phosphoproteins generated after activation of protein kinase C, both in cell-free conditions and in intact fibroblasts, are identical as judged by one and two-dimensional polyacrylamide slab gel electrophoresis and peptide mapping. Prolonged treatment of cells with phorbol esters causes a selective decrease in protein kinase C activity and prevents the stimulation of 80 K phosphorylation in intact fibroblasts. We now show that extracts from PBt_2-treated cultures fail to stimulate 80 K phosphorylation after the addition of the protein kinase C activators. This effect was due to the lack of protein kinase C activity since the addition of exogenous protein kinase C from mouse brain stimulated 80 K phosphorylation in both control and PBt_2-treated preparations. The 80 K phosphoprotein generated by activation of endogenous and exogenous protein kinase C yielded similar phosphopeptide fragments after peptide mapping by limited proteolysis. We conclude that the detection of changes in the phosphorylation of 80 K provides a useful approach to ascertain which extracellular ligands activate protein kinase C in intact cells.

Key words: growth control/phorbol esters/diacylglycerol/phosphoprotein

Introduction

Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) (Takai *et al.*, 1979; Kuo *et al.*, 1980; Kikkawa *et al.*, 1982), which is stimulated by unsaturated diacylglycerols (Takai *et al.*, 1979; Kishimoto *et al.*, 1980; Sano *et al.*, 1983) and serves as a major receptor for the tumour promoters of the phorbol ester family (for review, see Nishizuka, 1984), may play an important role in signalling a variety of cellular responses including cell growth. Accordingly, it is of importance to determine which mitogenic agents lead to activation of protein kinase C in intact, quiescent cells.

Recently, we proposed that a rapid increase in the phosphorylation of an 80 000 mol. wt. cellular protein (termed 80 K) reflects the activation of protein kianse C in intact cells (Rozengurt *et al.*, 1983a). Several lines of evidence support this conclusion. (i) Addition of biologically active phorbol esters to quiescent cells stimulates a rapid (15 s) phosphorylation of this protein. (ii) Stimulation of phospholipid breakdown leading to generation of diacylglycerol either by exogenous phospholipase C (Kent, 1979; Sleight and Kent, 1983) or by platelet-derived growth factor (PDGF) (Ross and Vogel, 1978) which activates endogenous phospholipase C (Habenicht *et al.*, 1981; Shier and Durkin, 1982; Rozengurt *et al.*, 1983b), also causes a rapid enhancement of 80 K phosphorylation (Rozengurt *et al.*, 1983a; Rodriguez-Pena and Rozengurt, 1985). (iii) The synthetic diacylglycerol 1-oleoyl-2-acetyl glycerol (OAG), which is biologically active when presented to intact platelets (Kaibuchi *et al.*, 1983; Rink *et al.*, 1983), stimulates the phosphorylation of the same 80 K protein in quiescent 3T3 cells (Rozengurt *et al.*, 1984). (iv) Prolonged pre-treatment of the cells with phorbol esters, which leads to a marked decrease in the number of specific phorbol ester binding sites (Collins and Rozengurt, 1982a, 1982b, 1984; Rozengurt and Collins, 1983) and to disappearance of protein kinase C activity measured in cell-free preparations (Rodriguez-Pena and Rozengurt, 1984; Rozengurt *et al.*, 1985), prevents the increase in 80 K phosphorylation elicited by all these diverse agents, namely, phorbol esters, phospholipase C, OAG and PDGF (Rozengurt *et al.*, 1983a, 1984). All these findings suggest that 80 K phosphorylation reflects the activation of protein kinase C in intact 3T3 cells.

Since a rapid activation of protein kinase C leading to an increase in phosphorylation of the 80 K cellular protein might be a significant event in the initiation of mitogenesis (Rozengurt *et al.*, 1983a, 1984, 1985; Rodriguez-Pena and Rozengurt, 1985), it was of importance to test directly the proposition that 80 K serves as a substrate of endogenous protein kinase C. Using a cell-free system we demonstrate here that addition of activators of protein kinase C, i.e., phosphatidylserine (PS), Ca^{2+}, and phorboldibutyrate (PBt_2) in the presence of [γ-^{32}P]ATP, stimulates the phosphorylation of an 80 K protein. This protein is similar to the 80 K phosphoprotein generated in intact cells as judged by one- and two-dimensional PAGE and peptide mapping. Further, prolonged treatment of the cells with PBt_2 which blocks 80 K phosphorylation in response to a subsequent addition of PBt_2 also abolishes the phosphorylation response elicited by addition of PBt_2, Ca^{2+} and PS to cell-free extracts. Although 80 K has not been identified yet, the detection of changes in its phosphorylation provides a novel approach to assess which mitogenic agents activate protein kinase C in intact fibroblastic cells.

Results

To identify potential protein substrates for protein kinase C in Swiss 3T3 cells, detergent-solubilized extracts of these cells were incubated in the presence of [γ-^{32}P]ATP under various conditions designed to activate this phosphotransferase. The pattern of phosphorylated endogenous proteins was examined by SDS-polyacrylamide slab gel electrophoresis (PAGE) and the protein bands containing ^{32}P were revealed by autoradiography of the dried gel. Figure 1 shows the effect of addition of PS, PBt_2 or both on endogenous protein phosphorylation in extracts of 3T3

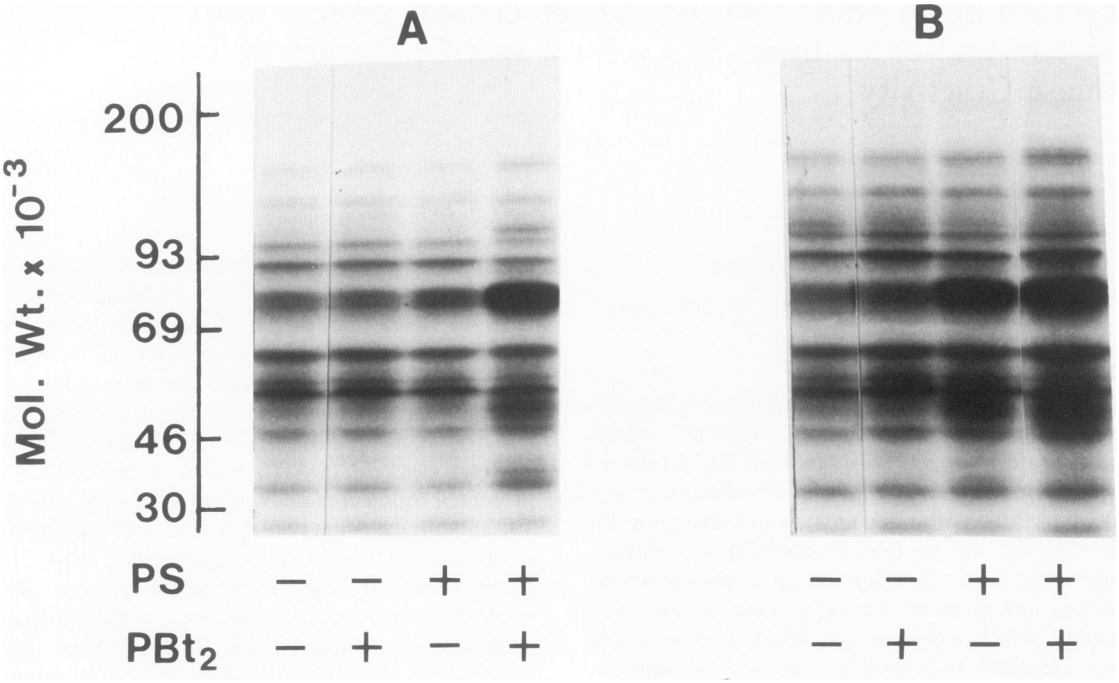

Fig. 1. Autoradiogram showing phosphorylation of proteins from detergent-solubilized extracts of quiescent cultures of Swiss 3T3 cells by endogenous protein kinase C. Cell-free extracts from quiescent cultures of Swiss 3T3 cells were prepared as described. Phosphorylation of endogenous proteins was carried out for 15 s at 30°C in the presence or absence of PS (80 μg/ml) and PBt_2 (100 ng/ml) as indicated. The concentration of $CaCl_2$ in **panels A** and **B** were 98 μM and 200 μM, respectively. EGTA was present at 100 μM. The concentrations of free Ca^{2+} calculated as described by Bartfai (1979) were 3.7 μM **(panel A)** and 100 μM **(panel B)**.

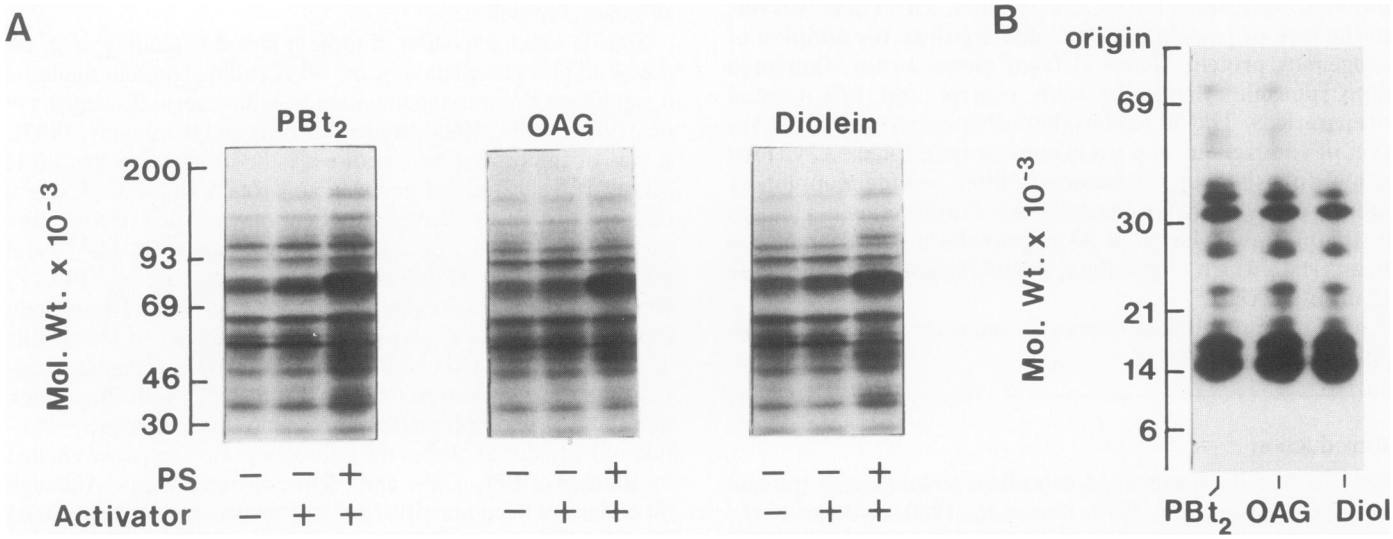

Fig. 2. Activation of protein kinase C '*in vitro*' by phorbol esters (PBt_2), 1 oleoyl-2-acetyl-glycerol (OAG) and 1,2 diolein (Diolein) stimulates the phosphorylation of a 80 000-dalton protein. **(A)** Detergent-solubilized extracts of Swiss 3T3 cells were incubated for 15 s at 30°C in the presence of 100 μM EGTA, 98 μM $CaCl_2$ with or without 80 μg/ml PS. The concentrations of PBt_2, OAG and 1,2 diolein were 0.1 μg/ml, 1 μg/ml and 1 μg/ml, respectively. **(B)** Cleveland map of 80 K phosphoprotein generated by activation of protein kinase C by either PBt_2, OAG or Diolein. The 80 K phosphoproteins generated in the presence of PS and activator shown in **A** were excised from the gel and digested with 0.05 μg V8 protease.

cells incubated in the presence of Ca^{2+} at two different concentrations. The reaction was terminated after 15 s. At low Ca^{2+} concentrations (3.7 μM), addition of PS or PBt_2 singly, failed to stimulate any significant phosphorylation (Figure 1A). In contrast, addition of both PS and PBt_2 resulted in a marked increase in the phosphorylation of several proteins but the main substrate was a protein that migrated with an apparent mol. wt. of 80 000 (termed 80 K). The effect of phorbol esters was specific; neither phorbol nor 12-tetradecanoate 13-acetate 4-O-methyl ether (MeTPA), which are biologically inactive analogues of PBt_2, had an effect, whereas 12-tetradecanoate 13-acetate (TPA) was as effective as PBt_2 in stimulating 80 K phosphorylation (Table I).

Phorbol esters, like diacylglycerols, stimulate protein kinase C by increasing the apparent affinity of the enzyme for Ca^{2+} (Kishimoto *et al.*, 1980; Castagna *et al.*, 1982; Kikkawa *et al.*, 1983). Hence, if activation of protein kinase C is responsible

Table I. Specific effect of phorbol esters on 80 K phosphorylation in Swiss 3T3 cell-free preparations

Addition	80 K phosphorylation (c.p.m. × 10^{-3})
–	390
Phorbol	325
MeTPA	375
TPA	2800
PBt_2	2850

Detergent-solubilized preparations from Swiss 3T3 cells were incubated for 15 s at 30°C in the presence of 80 μg/ml PS, 100 μM EGTA, 98 μM $CaCl_2$ and either phorbol 12-tetradecanoate 13-acetate 4-O-methyl ether (MeTPA) phorbol, 12-tetradecanoate 13-acetate (TPA) or PBt_2 at 100 ng/ml. The different incubation samples were analyzed by SDS-PAGE. The 80 K phosphoproteins were located by autoradiography of the dried gel and excised for counting the ^{32}P incorporated.

for increasing 80 K phosphorylation in cell-free extracts, the requirement for phorbol esters should be eliminated by increasing the concentration of Ca^{2+} in the incubation mixture. In accord with this, addition of PS in the presence of 100 μM Ca^{2+} was sufficient to stimulate 80 K phosphorylation (Figure 1B). Furthermore, the results shown in Figure 2 demonstrate that PBt_2 could be substituted either by 1,2 diolein, a diacylglycerol that activates the enzyme in cell-free preparations but not in intact cells (Kaibuchi *et al.*, 1983) or by the synthetic diacylglycerol OAG which activates protein kinase C both in cell-free preparations and in intact 3T3 cells (Rozengurt *et al.*, 1984, 1985). Peptide mapping of the 80 K phosphoproteins generated in response to PBt_2, OAG or diolein after limited proteolysis with the *Staphylococcus aureus* V8 protease showed identical phosphopeptide fragments (Figure 2B). In contrast, addition of either cAMP (10 μM) or cGMP (10 μM) to the cell-free system failed to stimulate 80 K phosphorylation.

Figure 3 shows the incorporation of ^{32}P into 80 K as a function of time. The reaction was linear for 15 s; in the presence of PS, Ca^{2+} and PBt_2 the rate of incorporation was 5-fold higher than in the absence of the activators. Peptide mapping shows that the same 80 K phosphoprotein is phosphorylated at various times of incubation (Figure 3, bottom).

Recently, we described that phorbol esters and OAG, which activate protein kinase C, stimulate the phosphorylation of an 80 000-dalton cellular protein in intact Swiss 3T3 cells. To test whether the 80 K phosphoprotein generated in cell-free extracts by activation of protein kinase C is closely related to the 80 K phosphoprotein obtained in intact cells, we examined these phosphoproteins by analysis in two-dimensional PAGE. Intact, quiescent cultures of Swiss 3T3 cells were labeled with $^{32}P_i$ and treated with or without PBt_2 for 1 min while detergent-solubilized extracts from parallel cultures were incubated with [γ -^{32}P]ATP in the absence or presence of PS, Ca^{2+} and PBt_2 for 15 s. Then, the phosphoproteins generated '*in vivo*' and '*in vitro*' were subjected to two-dimensional PAGE. As shown in Figure 4, the 80 K phosphoproteins generated '*in vivo*' and '*in vitro*' in response to PBt_2 co-migrated at an identical position as an acidic protein (pI 5). The possibility that the same 80 K protein is phosphorylated in cell-free extracts and in intact cells in response to PBt_2 was further substantiated by phosphopeptide mapping using V8 protease (Figure 5).

Treatment of cells with phorbol esters leads to progressive 'down-modulation' of phorbol ester receptors in intact cells (Col-

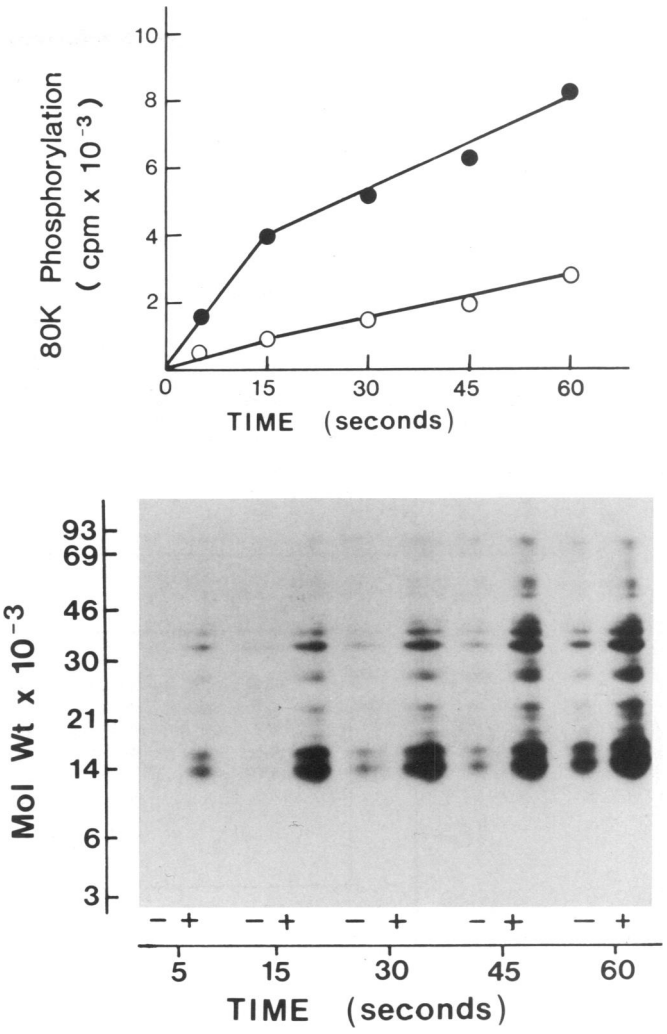

Fig. 3. Time course of 80 K phosphorylation by protein kinase C '*in vitro*'. **Top:** detergent solubilized extracts of quiescent 3T3 cells were prepared as described. The phosphorylation assays were performed with 100 μM EGTA at 30°C in the absence (○) or presence (●) of PS, PBt_2 and 30.5 μM $CaCl_2$. The reaction was stopped at different times and the samples analysed on a polyacrylamide slab gel. The 80 K phosphoproteins were located by autoradiography of the dried gel and excised for counting the ^{32}P incorporated. **Bottom:** peptide mapping of the 80 K phosphoproteins generated in the absence (−) or the presence (+) of PS, Ca^{2+} and PBt_2 after different incubation times. The amount of V8 protease used was 0.05 μg/well.

lins and Rozengurt 1982a, 1982b, 1984), to disappearance of protein kinase C activity in cell-free preparations (Rodriguez-Pena and Rozengurt, 1984; Rozengurt *et al.*, 1985) and blocks the stimulation of 80 K phosphorylation in response to PBt_2 (Rozengurt *et al.*, 1983a) or OAG (Rozengurt *et al.*, 1984) in intact cells. If PS and PBt_2 stimulate 80 K phosphorylation in the cell-free system by activating endogenous protein kinase C, prolonged pre-treatment of the cells with PBt_2 prior to preparation of cell-free extracts should block the stimulation of 80 K phosphorylation produced by PS, and PBt_2. Figure 6 shows that treatment of the cells for 40 h with PBt_2 blocked the enhancement of 80 K phosphorylation produced by activation of protein kinase C in the cell-free extract. If the prolonged pre-treatment with PBt_2 causes a selective decrease in protein kinase C activity and thereby prevents 80 K phosphorylation, it should be possible to reconstitute the 80 K phosphorylating system using extracts

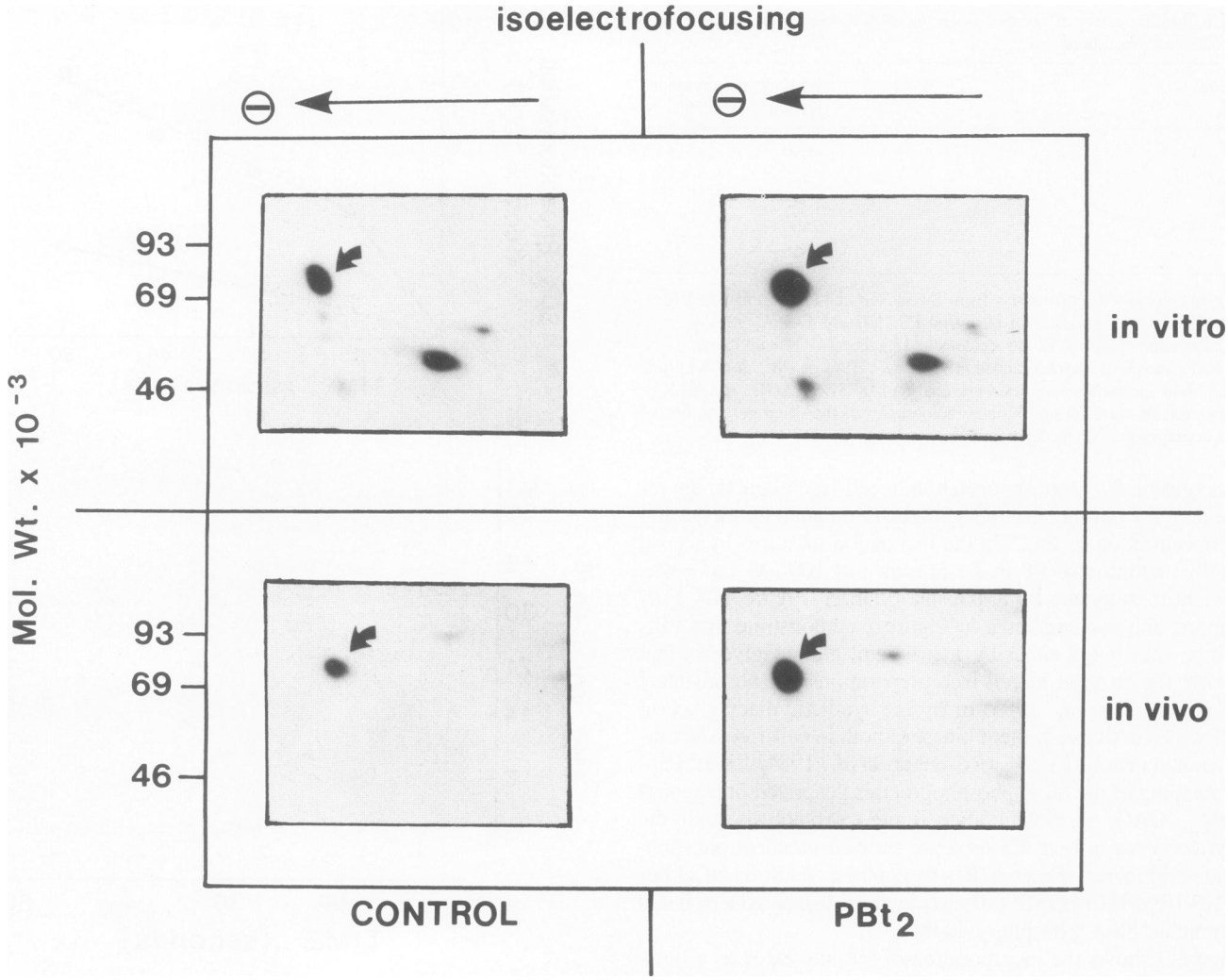

Fig. 4. Resolution of the 80 K phosphoproteins generated in cell-free extracts of Swiss 3T3 cells and in intact quiescent fibroblasts by two-dimensional PAGE. The phosphorylation '*in vitro*' of detergent-solubilized extract from quiescent fibroblasts was carried out as described, in the absence (control) or the presence (PBt_2) of 80 μg/ml PS, 98.5 μM $CaCl_2$, 100 μM EGTA and 100 ng/ml PBt_2 for 15 s at 30°C. The phosphorylation of 80 K in intact quiescent Swiss 3T3 cells labelled with ^{32}P ('*in vivo*') was examined after exposure to either 100 ng/ml PBt_2 or an equivalent volume of solvent (control) for 1 min. In the first dimension, phosphoproteins were separated by isoelectric focussing from the right (basic) to the left (acidic) and in the second dimension by SDS-PAGE (10% polyacrylamide). The position of the 80 K phosphoproteins is indicated by an arrow.

of PBt_2-treated cells supplemented with exogenous protein kinase C. To test this possibility, control and PBt_2-treated extracts were incubated in the absence or presence of partially purified mouse brain protein kinase C and the incorporation of ^{32}P into 80 K was measured. In accord with the results presented in Figure 6, addition of PS, PBt_2 and Ca^{2+} failed to stimulate 80 K phosphorylation in extracts from PBt_2-treated cells (Figure 7B). The salient feature of the results presented in Figure 7 is that addition of protein kinase C stimulates 80 K phosphorylation in both control and pre-treated cells. The 80 K generated by activation of endogenous or exogenous protein kinase C yielded similar phosphopeptide fragments when subjected to proteolysis with V8 protease (Figure 7C). As a control, we verified that incubation of mouse brain protein kinase C at the concentrations used in Figure 7 in the presence of [γ-^{32}P]ATP but in the absence of cellular extract did not exhibit any 80 K phosphorylation. This preparation produced a faint band that migrated with an apparent mol. wt. of 86 000 and was clearly separated from the 80 K protein.

Discussion

The results presented here demonstrate that incubation of detergent-solublized extracts of Swiss 3T3 cells under a variety of conditions designed to stimulate endogenous protein kinase C causes rapid phosphorylation of an 80 K protein. In the presence of PS and low concentrations of Ca^{2+} (3.7 μM), addition of various effectors which increase the apparent affinity of protein kinase C for Ca^{2+} (Kishimoto *et al.*, 1980; Castagna *et al.*, 1982) markedly stimulated the incorporation of ^{32}P into 80 K. These include the diacylglycerol 1,2, diolein which stimulates protein kinase C activity in cell-free extracts (Takai *et al.*, 1979), the synthetic diacylglycerol OAG, which activates protein kinase C in cell-free extracts as well as in intact cells (Kaibuchi *et al.*, 1983; Rink *et al.*, 1983; Rozengurt *et al.*, 1984) and the potent tumour promoters of the phorbol ester family such as PBt_2 and TPA, which bind and activate homogeneous preparations of protein kinase C (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983; Parker *et al.*, 1984). The requirement of these effectors to stimulate 80 K phosphorylation was by-passed by increasing

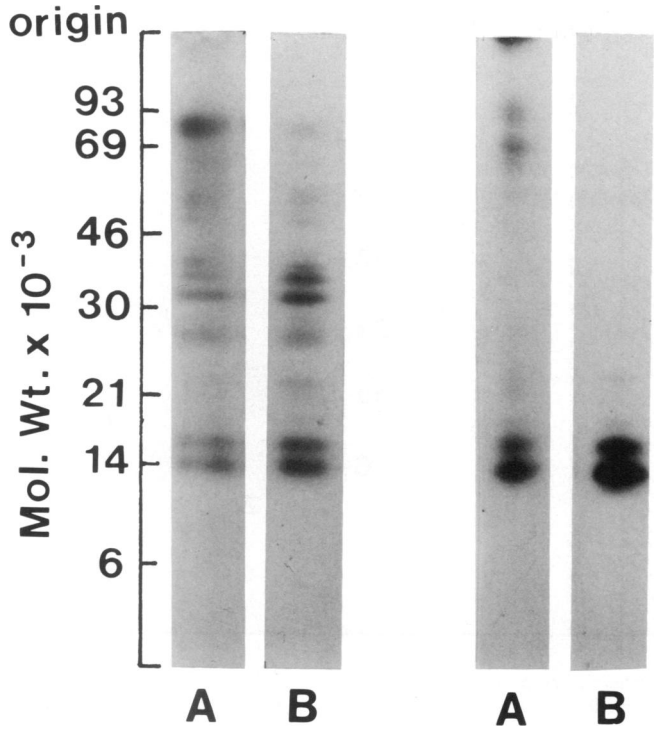

Fig. 5. V8 protease digestion of 80 K phosphoproteins generated in intact 3T3 cells treated with PBt_2 **(A)** or in cell-free preparations supplemented with Ca^{2+}, PS and PBt_2 **(B)**. The phosphorylation of 80 K in intact fibroblasts labelled with ^{32}P was stimulated by 100 ng/ml PBt_2 for 1 min. The reaction was terminated by the addition of SDS sample buffer, and the samples were subjected to SDS-PAGE. The phosphorylation of 80 K protein from detergent-solubilized extracts of quiescent fibroblasts was carried out as described in Materials and methods. The 80 K phosphoproteins were re-run in a 15% acrylamide slab gel in the presence of 0.05 μg (left) or 1 μg V8 protease (right). **(A)** '*in vivo*' phosphorylation induced by PBt_2; **(B)** '*in vitro*' phosphorylation by direct activation of protein kinase C.

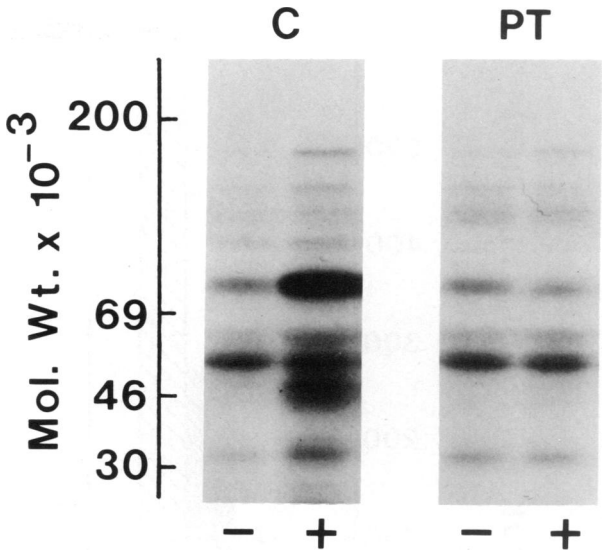

Fig. 6. Effect of pre-treatment with PBt_2 on the phosphorylation of 80 K in cell-free extracts. Quiescent cultures of Swiss 3T3 cells were exposed to 200 ng/ml of PBt_2 for 40 h. After this time, detergent-solubilized extracts from the same number of control (C) and PBt_2-treated (PT) dishes were prepared. The phosphorylation assay was performed with 0.5 mM EGTA, 0.45 mM $CaCl_2$, at 30°C for 15 s in the absence (−) or the presence (+) of 100 ng/ml PBt_2 and 80 μg/ml PS.

the concentration of Ca^{2+} in the incubation mixture (from 3.7 to 100 μM). We verified that the 80 K phosphoproteins generated in response to each effector gave similar phosphopeptide fragments when subjected to peptide mapping by limited proteolysis, with V8 protease. Other second messengers (i.e., Ca^{2+}, cAMP or cGMP) added to cell-free extracts failed to stimulate 80 K phosphorylation. These findings provide direct evidence that 80 K can serve as a substrate of protein kinase C.

We have previously reported that addition of either phorbol esters (Rozengurt *et al.*, 1983a) or OAG (Rozengurt *et al.*, 1984) which both activate protein kinase C in intact cells, lead to a rapid phosphorylation of an 80 000-dalton cellular protein in Swiss 3T3 cells. It was, therefore, of great interest to test whether the same 80 K phosphoproteins are generated by activation of protein kinase C in intact cells ('*in vivo*') and in cell-free extracts ('*in vitro*'). Analysis using one- and two-dimensional electrophoresis and peptide mapping strongly suggests that the 80 K phosphoprotein generated both '*in vivo*' and '*in vitro*' are identical. These findings support the hypothesis that 80 K phosphorylation in intact cells reflects, at least in part, changes in protein kinase C activity.

Previous work has shown that prolonged treatment of 3T3 cells with phorbol ester causes a progressive decline in the number of phorbol ester binding sites (Collins and Rozengurt, 1982a, 1982b, 1984), a decrease in the activity of protein kinase C measured in cell-free extracts (Rodriguez-Pena and Rozengurt, 1984), and desensitizes the cells to further biological effects of PBt_2 (Collins and Rozengurt, 1984; Rozengurt *et al.*, 1983a, 1984, 1985). Here we show that prolonged treatment with PBt_2 of intact Swiss 3T3 cells prior to the preparation of the cell-free extracts, completely prevented the stimulation of 80 K phosphorylation caused by the addition of protein kinase C activators (i.e., PS, Ca^{2+} and PBt_2). This result is in accord with the disappearance of 80 K phosphorylation in intact cells and further substantiates the proposition that the phosphorylation of 80 K reflects the activation of protein kinase C.

If prolonged pre-treatment with PBt_2 selectively reduces protein kinase C, it should be possible to restore the ability of the cell-free system to phosphorylate 80 K by adding exogenous protein kinase C. The results presented in Figure 7 are in line with this prediction. This finding has several important implications: (i) it provides further evidence that 80 K phosphorylation is promoted by protein kinase C; (ii) it rules out the possibility that the failure of PS and PBt_2 to stimulate 80 K phosphorylation in cell-free extracts prepared from PBt_2-treated cells is due merely to a low rate of turnover of the 80 K phosphoprotein generated during the early stages of the treatment with PBt_2, and (iii) it strongly suggests that protein kinase C and 80 K are separate proteins, although direct confirmation of this hypothesis will require further experimental work.

Recent evidence demonstrates that activation of protein kinase C by diacylglycerol and phorbol esters acts as a mitogenic signal for quiescent 3T3 cells (Rozengurt *et al.*, 1984, 1985). Hence the stimulation of this phosphotransferase system may play a fundamental role in effecting the proliferative response elicited by certain growth factors. A crucial step to evaluate this hypothesis requires a procedure to assess which growth factors increase the activity of protein kinase C in intact cells. The results presented here provide direct evidence that changes in the phosphorylation of an acidic 80 000-dalton protein constitutes a specific

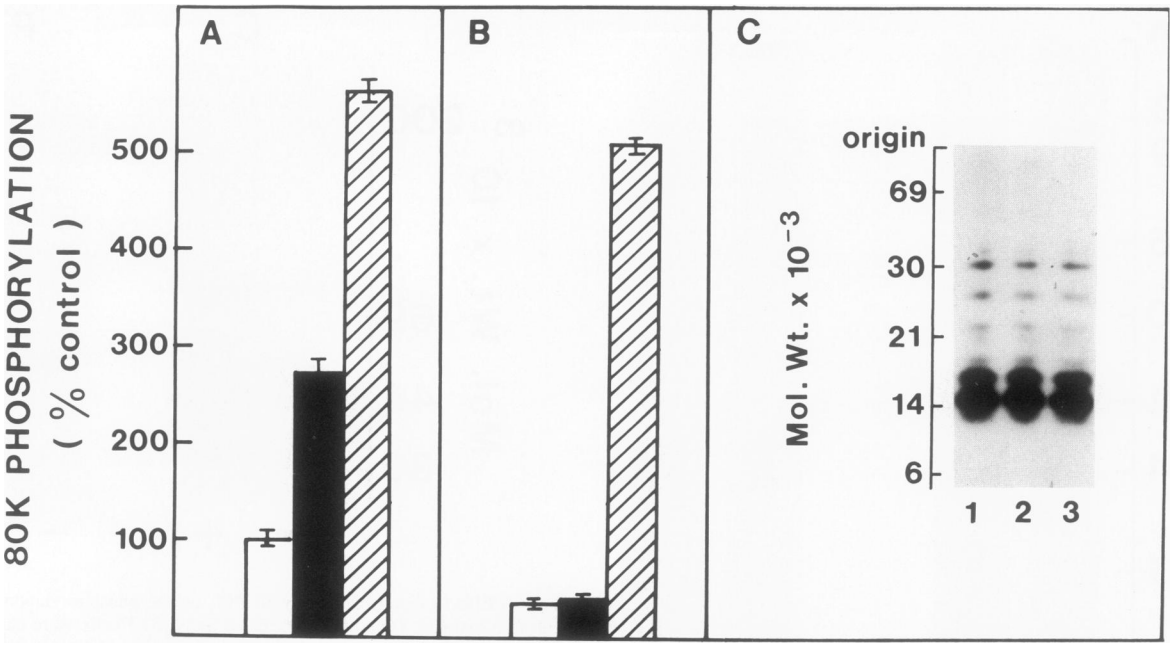

Fig. 7. Effect of the addition of protein kinase C from mouse brain on the 80 K phosphorylation in cell-free extracts from control and PBt_2-treated cultures. Quiescent cultures of Swiss 3T3 cells were treated with 200 ng/ml PBt_2 for 40 h. Detergent-solubilized extracts from control **(panel A)** and PBt_2-treated **(panel B)** dishes were prepared. The phosphorylation assay was performed with 0.5 mM EGTA, 0.45 mM $CaCl_2$ at 30°C for 15 s under the following conditions: no addition (open bars), 80 μg/ml PS and 100 ng/ml PBt_2 (closed bars), 80 μg/ml PS, 100 ng/ml PBt_2 and 2.4 μg of protein kinase C from mouse brain (sp. act. 250 nmol ^{32}P/min/mg protein) (cross-hatched bars). The samples were run in 8% acrylamide gels and the 80 K bands from the dried gel were excised and counted. The figures are expressed as % of the ^{32}P incorporated into 80 K of extracts incubated in the absence of the protein kinase C activators PS and PBt_2 (691 c.p.m. ± 98) and represent the mean of two experiments. The activity of the mouse brain protein kinase C depended on the presence of PS and PBt_2; the phosphorylation of 80 K in the absence of PS and PBt_2 was 981 ± 110 c.p.m. **Panel C:** peptide mapping of the 80 K phosphoproteins generated in control and in PBt_2-treated extracts shown in **A** and **B**. Control extract with PS and PBt_2 **(lane 1)**, control extract with PS, PBt_2 and mouse brain protein kinase C **(lane 2)** and extract from PBt_2-treated cells in the presence of PS, PBt_2 and mouse brain protein kinase C **(lane 3)** . The amount of V8 protease used was 0.05 μg/well.

marker to ascertain which extracellular growth factors activate protein kinase C in intact fibroblastic cells. Further understanding of the nature of this protein and the effects of phosphorylation by protein kinase C may be of relevance for understanding the initial events in the stimulation of cell proliferation.

Materials and methods

Cell cultures

Stock cultures of Swiss 3T3 cells (Todaro and Green, 1963) were propagated in 90-mm Nunc dishes in Dulbecco's modified Eagle's (DME) medium containing 10% fetal bovine serum in a humidified atmosphere of 10% CO_2:90% air at 37°C. For experimental use, the cells were subcultured into either 33-mm or 90-mm Nunc dishes in DME medium with 10% fetal bovine serum for 7 days. The 90-mm cultures were re-fed afte 2 days' growth and incubated for a further 5 days. After this time, the cells were arrested in G_1, G_0 phase of the cell cycle, as judged by the fact that < 1% of the cells became autoradiographically labelled after 40 h exposure to [^{3}H]thymidine (Dicker and Rozengurt, 1980; Rozengurt and Heppel, 1975).

Preparation of cell-free extracts

Quiescent cultures of Swiss 3T3 cells in 90-mm dishes were washed twice with ice-cold 0.15 M NaCl. Each dish was scraped with a rubber policeman into 0.7 ml of homogenization buffer which contained 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 0.5% Triton-X-100. The cell suspensions were pooled and disrupted by 20 strokes with a Dounce homogenizer and the detergent-solubilized extract was centrifuged at 100 000 *g* for 1 h. The supernatant was fractionated with $(NH_4)_2SO_4$ (33 – 70% saturation) and the precipitated protein fraction was resuspended at 10 mg protein/ml in 20 mM Tris-HCl pH 7.5, 1 mM EGTA, 50 mM 2-mercaptoethanol, 25% glycerol, and stored at −70°C. The preparation was dialyzed for 5 h against 5000 volumes of the same buffer before being used in the phosphorylation assays. All the procedures were carried out at 4°C.

Phosphorylation 'in vitro'

The preparation of detergent-solubilized extracts from Swiss 3T3 cells were dialyzed and phosphorylated at 30°C using the following reaction conditions: 20 mM Tris-HCl pH 7.5, 7 mM magnesium acetate, 2.5% glycerol, 5 mM 2-mercaptoethanol, 100 μM EGTA, 15 μM [γ-^{32}P]ATP (3 Ci/mmol) and protein (1 mg/ml). PS (80 μg/ml), PBt_2 (100 ng/ml) and $CaCl_2$ at different concentrations were added as indicated in each experiment. The reaction was started by addition of [γ-^{32}P]ATP and terminated after 15 s by the addition of two times concentrated SDS-sample buffer (containing 3% SDS, 5% glycerol, 10 mM Tris-HCl, pH 7.5 and 2% 2-mercaptoethanol). The samples were immediately heated at 100°C for 10 min prior to analysis by SDS-PAGE.

Phosphorylation in intact cells

Quiescent Swiss 3T3 cells in 33-mm Nunc dishes were washed twice with DME medium without phosphate and incubated with this medium containing 200 μCi/ml of carrier free $^{32}P_i$ at 37°C for 4 h to label the endogenous ATP pool. Then, biologically active phorbol ester or an equivalent volume of solvent were added for 1 min. The reaction was stopped by removing the medium and rapidly washing the cultures twice with ice-cold Tris-saline solution (0.15 M NaCl, 20 mM Tris-HCl pH 7.5). The cells were immediately extracted with 5% trichloroacetic acid at 4°C for 20 min. The acid-soluble pools were removed; the precipitated protein was washed twice with Tris-saline and dissolved in 100 μl SDS sample buffer heated at 100°C. After 2 min, the samples were placed in a boiling water bath for 10 min prior to resolution by gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE)

Slab gel electrophoresis was performed using a 5 – 15% acrylamide gradient and 0.1% SDS by the method of Laemmli (1970). After electrophoresis, the slabs were stained, destained and dried down onto paper for radiography with Fuji X-ray film (Fuji Photo Film Co. Ltd., Japan). Two dimensional-PAGE was carried out as described by O'Farrell (1975), using isoelectric focusing (1.4% LKB ampholytes pH 5 – 7 plus 0.6% LKB ampholytes pH 3.5 – 10) in the first dimension and SDS-PAGE (10% polyacrylamide) in the second dimension.

Peptide mapping by limited proteolysis

One-dimensional peptide mapping (Cleveland *et al.*, 1977) was performed using *S. aureus* V8 protease at different concentrations. The proteins for digestion were excised from dried SDS-polyacrylamide slab gels after fixation and autoradiography. Individual gel bands containing the same amount of counts were rehydrated for 2 h in a solution containing 125 mM Tris-HCl pH 6.8, 0.1% SDS,

1 mM EDTA and placed into separate wells of a discontinuous SDS-PAGE (15% acrylamide). The gel bands were overlayed with 100 μl of the same buffer containing 15% (w/v) glycerol and different amounts of *S. aureus* V8 protease. Electrophoresis was carried out at 60 V.

Protein kinase C

Protein kinase C was partially purified from mouse brain by the method of Walsh *et al.* (1984). Briefly, 25 – 50 fresh mouse brains were homogenized in 20 mM Tris-HCl buffer pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol and 0.5% Triton-X-100. The homogenate was centrifuged at 100 000 *g* for 1 h. The supernatant was precipitated with $(NH_4)_2SO_4$ (30 – 70% saturation) and the pellet protein re-dissolved in a minimum volume of 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol and dialysed (18 h) against 2 × 4 l of the same buffer. The sample was adjusted to 2 mM $CaCl_2$, 2 mM $MgCl_2$ and applied to a phenyl-Sepharose CL-4B column previously equilibrated with 0.1 mM $CaCl_2$, 20 mM Tris-HCl pH 7.5 and 1 mM dithiothreitol. After different washing steps the column was eluted with 20 mM Tris-HCl buffer pH 7.5 containing 1 mM EGTA and 1 mM dithiothreitol. Aliquots (30 μl) of each fraction were assayed for protein kinase C activity using histone III-S as substrate as described previously (Rodriguez-Pena and Rozengurt, 1984). The protein kinase C preparation had a specific activity of 250 nmol ^{32}P/min/mg protein.

Materials

Diolein, PBt_2 and PS were obtained from Sigma Chemical Co. (St. Louis, MO), [γ-^{32}P]ATP and $^{32}PO_4$ carrier-free were from the Radiochemical Centre (Amersham, UK) and *S. aureus* V8 protease was from Miles Laboratories. Synthesis of OAG was as described by Buchnea (1971) and kindly provided by Dr M. Coombs.

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