The phosphorylation of protein kinase C as a potential measure of activation

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As a means of determining the role of protein kinase C in the signal transduction from novel growth factors and hormones, we investigated the effects of well-characterized agents on the phosphorylation state of protein kinase C itself. These studies show that agents that stimulate protein kinase C either directly (phorbol esters) or indirectly through phosphatidylinositol breakdown (platelet-derived growth factor) induce an increase in the phosphorylation state of the kinase. By contrast, epidermal growth factor, which does not stimulate protein kinase C in fibroblasts, does not increase the phosphorylation state of protein kinase C, but leads to a decrease. The data suggest that the phosphorylation state of protein kinase C is dynamically controlled and can be used to provide evidence of protein kinase C activation.

INTRODUCTION

The phosphorylation of proteins is an important mechanism by which many cellular processes are regulated [reviewed by Cohen (1982) and Nestler *et al.* (1984)]. Many protein kinases have been described, and among these much recent interest has centred on protein kinase C (PKC), which is thought to play a pivotal role in many cellular control processes. This protein kinase is a serine/threonine-specific kinase, dependent on Ca^{2+} , phospholipid and diacylglycerol for its activity (reviewed by Nishizuka, 1986). PKC is also stimulated by phorbol esters, through their ability to mimic the effects of diacylglycerol; PKC has been defined as the major phorbol ester receptor in cells (see Ashendel, 1985).

The cDNA cloning of PKC has revealed that there are at least seven polypeptide members of this family (reviewed by Nishizuka, 1988). The biochemical characterization of their activities is a major task and should lead to a clearer understanding of the roles of these regulatory enzymes. However, in order to address the activation of these enzymes *in vivo*, particularly in cells where multiple PKC genes are expressed, it will be necessary to develop novel methodologies.

To date, activation of PKC has most frequently been monitored through an assessment of 'translocation'; but, although useful, this phenomenon is difficult to reproduce in many situations (discussed by Woodgett *et al.*, 1987). Another approach to the question of PKC activation has been to define possible cellular targets for the enzyme and monitor their changing phosphorylation states. For example, Rozengurt *et al.* (1983) reported that the activation of PKC is reflected by increased phosphorylation of an 80 kDa cellular protein (termed 80K) in quiescent 3T3 cells. Enhanced phosphorylation of 80K in response to phorbol esters has also been demonstrated in several other cell lines, such as 3T3-preadipocytes (Blackshear *et al.*, 1985) and in C3H 10T_{1/2} mouse fibroblasts (Mahadevan *et al.*, 1987). This type of ap-

proach has been particularly useful in circumstances where targets (direct or indirect) are relatively abundant proteins. In general, it is not always possible to define such major targets, and furthermore such analyses are complicated by the multi-site phosphorylation of many regulatory proteins and the co-expression of different PKC subtypes.

Protein kinase C not only phosphorylates a number of proteins both *in vivo* and *in vitro* (Woodgett *et al.*, 1987) but also, like many kinases, undergoes autophosphorylation, in which multiple sites are phosphorylated on serine and threonine residues (Huang *et al.*, 1986a; Parker *et al.*, 1986; Woodgett *et al.*, 1987). In order to assess whether the phosphorylation state of PKC may itself be a useful marker of PKC activation *in vivo*, we have examined whether there are quantitative or qualitative changes in the phosphate content of PKC in a primary human fibroblast cell treated with agents that are thought either to activate PKC directly (TPA) or through phosphatidylinositol turnover (PDGF) or agents that are not apparently coupled to this pathway in these cells (EGF). This analysis shows that the phosphorylation state of PKC does increase in response to activators, and this may be a useful tool for assessing PKC activation. The possibility that these changes may have functional consequences is also discussed.

MATERIALS AND METHODS

Materials

HFF (human foreskin fibroblast) cells were obtained from the ICRF Cell Production Unit. Dulbecco's modified Eagle's minimal essential medium (DMEM), DMEM without phosphate, foetal-calf serum and penicillin/streptomycin were supplied by Gibco, Uxbridge, Middx., U.K. [³²P]P_i was purchased from Amersham International, Amersham, Bucks., U.K. [γ -³²P]ATP was purchased from NEN, Stevenage, Herts., U.K. TPA, Hepes,

Abbreviations used: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's minimal essential medium; HFF cells, human foreskin fibroblast cells.

EDTA, Triton X-100, DL-dithiothreitol and SDS were from Sigma Chemical Co., Poole, Dorset, U.K. Monoclonal C5 antibody and competing peptide were as described elsewhere (Young *et al.*, 1988). Pig PDGF was purchased from R & D Systems, Minneapolis, MN, U.S.A. EGF was kindly provided by Dr C. Greenfield (Ludwig Institute, London).

Cell culture and labelling

HFF cells, passages 11–25, were grown in 150 mm-diam. dishes (Nunc) in 30 ml of DMEM, supplemented with 10% (v/v) foetal-calf serum and penicillin (1000 units/ml)/streptomycin (100 µg/ml). In order to allow the cells to quiesce, the medium was changed to DMEM supplemented with 1% foetal-calf serum and penicillin/streptomycin 3 days before either radiolabelling or harvesting of the cells. Before addition of radiolabel, cells were washed twice in 10 ml of phosphate-free DMEM and then incubated in 10 ml of phosphate-free DMEM containing 2 mCi of [³²P]P_i for 16–20 h. Subsequently cells were treated with TPA (4, 40 or 400 nM for 5, 15 or 60 min), PDGF (100 ng/ml for 5 or 20 min) or EGF (42 nM for 5 or 20 min) as indicated in the Figure legends. TPA was dissolved in dimethyl sulphoxide and added to the cells to give a final concentration of < 0.01% dimethyl sulphoxide.

Immunoprecipitation of PKC from labelled cells

Cells were washed twice with ice-cold Tris-buffered saline (0.15 M-NaCl/20 mM-Tris/HCl, pH 7.5) and lysed in 250 µl of boiling SDS sample buffer (Laemmli, 1970). The DNA was sheared by forcing the extract through a 21-gauge needle. The sample was then boiled for 5 min, centrifuged briefly (Microfuge, 2 min at 10000 rev./min) and the supernatant diluted to 1 ml with $\frac{4}{3} \times$ concentrated buffer A (20 mM-sodium phosphate, pH 7.2, 50 mM-NaF, 5 mM-EDTA, 1% Triton X-100, 0.1 mM-sodium orthovanadate, 25 µg of leupeptin/ml, 50 µg of phenylmethanesulphonyl fluoride/ml, 10 mM-benzamide, 10 mM-*p*-nitrophenol phosphate, 10 µg of aprotinin/ml, 1 mg of ovalbumin/ml and 1% deoxycholate). The supernatants were incubated with 60 µg of C5 antibody for 6 h at 4 °C (where present, competing peptide was preincubated with antibody for > 10 min at 4 °C before use). Immunocomplexes were removed on fixed *Staphylococcus aureus* (tumbled for 45 min at 4 °C), then washed three times with buffer A (without ovalbumin), and washed once with distilled water. Finally the pellets were resuspended in 15 µl of Laemmli sample buffer and electrophoresed on 8%-polyacrylamide gels in the presence of SDS. Gels were fixed, stained with Coomassie Brilliant Blue (Sigma) to detect protein standards, dried and autoradiographed at –80 °C by using Kodak X-ray film (SB5). Gel pieces were cut out and counted for radioactivity. Background regions were also cut out and counted for each lane; this has been subtracted from the PKC specific radioactivity.

Two-dimensional tryptic-peptide mapping

Dried gel pieces containing phosphorylated PKC were swollen in 50 mM-NH₄HCO₃ buffer, extracted four times in 100% acetone and washed three times in 50 mM-NH₄HCO₃ buffer. The gel pieces were then incubated in 0.5 ml of 50 mM-NH₄HCO₃ buffer with 50 µg of trypsin for 24 h, with tumbling at room temperature. The supernatant was removed, and the gel pieces were washed

with 0.5 ml of 50 mM-NH₄HCO₃ buffer for 1 h at room temperature. The supernatants were combined and a further 50 µg of trypsin was added; this was then incubated at 37 °C for 24 h. This procedure gave a recovery of 70–85% of the radioactivity. The digest was dried down, resuspended in 1 ml of distilled water, and re-dried; this was repeated twice more, and finally the pellet was resuspended in 15 µl of 1 M-acetic acid. Samples were loaded on to a t.l.c. plate. The first dimension was run on flat-bed apparatus in pyridine/acetic acid/water (1:10:189, by vol.) buffer, pH 3.5, for 20 min at 50 mA. The plates were allowed to dry and then subjected to the second-dimension ascending chromatography in pyridine/acetic acid/water/butan-1-ol (15:3:12:10, by vol.) which was allowed to run until the front was 2 cm from the top of the plate (about 2.5 h). The plates were air-dried and autoradiographed at –80 °C by using Amersham Hyper-film.

Measurement of PKC activity in partially purified cell extracts

Cell-free extracts were prepared as described by Rodriguez-Pena & Rozengurt (1984), except that the homogenization buffer did not contain 0.5% Triton X-100. The supernatants from the extraction were removed, fractionated with (NH₄)₂SO₄ (Rodriguez-Pena & Rozengurt, 1984) and assayed for PKC activity (Marais & Parker, 1989) with histone III-S as a substrate. The pellets were then extracted in homogenization buffer containing 0.5% Triton X-100 for 20 min at 4 °C, and centrifuged at 100000 *g* for 5 min; the supernatant was then fractionated with (NH₄)₂SO₄ (Rodriguez-Pena & Rozengurt, 1984), and PKC activity was assayed in the membrane fraction (Marais & Parker, 1989) with histone III-S as substrate.

Phosphorylation of the 80K PKC substrate

HFF cells were cultured and labelled essentially as described above, except that 90 mm-diam. Nunclon dishes were used and cells were labelled with 0.2 mCi of [³²P]P_i in 2 ml of phosphate-free DMEM. Subsequently cells were treated with either PDGF (100 ng/ml for 1, 5 or 20 min) or TPA (400 nM for 1, 5 or 20 min) as indicated in Fig. 6 legend. Cells were washed twice in ice-cold Tris-buffered saline and lysed in 250 µl of boiling SDS sample buffer (Laemmli, 1970). The DNA was sheared by forcing the extract through a 21-gauge needle. The sample was then boiled for 5 min, centrifuged briefly, and a 50 µl sample was electrophoresed on 10%-polyacrylamide gels in the presence of SDS. Gels were fixed, stained with Coomassie Brilliant Blue (Sigma) to detect protein standards, dried and autoradiographed at –80 °C by using Kodak X-ray film (SB5).

RESULTS

Initial studies focused on the phosphorylation state of PKC in the transformed glial cell line 328, which expresses high levels of PKC- α (Stabel *et al.*, 1987). However it was found that direct activation of PKC in these proliferating cells with TPA produced only a small increase in phosphorylation (a 20% increase above controls). This poor effect was considered to be due to the constitutive growth of these cells, since growth studies showed that it was not possible to obtain 328 cells in a quiescent state. In order to overcome this problem, HFF cells were

Fig. 1. Effect of TPA concentration on [^{32}P]P_i incorporation into PKC

PKC was immunoprecipitated from HFF-cell lysates with the antibody C5 as described in the Materials and methods section. Immunocomplexes were boiled in Laemmli sample buffer and subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue to reveal the PKC polypeptide at 79 kDa. From left to right, cells were treated with different concentrations (nM) of TPA for 60 min as indicated on the Figure. The right-hand lane shows high-molecular-mass standards (in descending order 200, 97.4 and 66.2 kDa).

chosen, as it was possible to arrest these primary cells in G₀ phase (results not shown).

Treatment of quiescent HFF cells with 400 nM-TPA, which directly activated PKC, resulted in a marked increase in ^{32}P incorporation into PKC immunoprecipitated from these cells (400 % compared with PKC immunoprecipitated from control cells; see Fig. 1). The increased phosphorylation of PKC was found to be dependent on the concentration of TPA, being highest at the highest concentration of TPA employed (400 nM) (Fig. 1). This increased phosphorylation was also time-dependent, reaching a maximum response at 60 min for 400 nM-TPA (Fig. 2, and see below). However, prolonged treatment of cells with 400 nM-TPA resulted in a marked decrease in the amount of immunoprecipitated ^{32}P -labelled PKC (Fig. 3). This is due to down-regulation of PKC; chronic exposure of cells to phorbol esters resulted in a loss of the kinase through proteolysis (See Young *et al.*, 1987).

In order to examine whether the increased incorporation of ^{32}P into immunoprecipitable PKC observed in HFF cells treated with TPA was paralleled by stimulation of phosphatidylinositol turnover, HFF cells were treated with PDGF. This also resulted in a stimulation of [^{32}P]P_i incorporation into PKC (Table 1). By contrast with PDGF, inositol lipid breakdown is not stimulated by EGF in fibroblasts (Besterman *et al.*, 1986), and no increase in the phosphate content of PKC was observed after EGF treatment of quiescent HFF cells (Table 1).

Fig. 2. Time-dependent effect of TPA on the phosphorylation of PKC

PKC was immunoprecipitated as described in the legend to Fig. 1. From left to right; immunoprecipitation from control cells, immunoprecipitation from control cells in the presence of competing peptide, high-molecular-mass standards (in descending order 200, 97.4 and 66.2 kDa), and immunoprecipitations from HFF cells treated with 400 nM-TPA for the times (min) indicated on the Figure.

Fig. 3. Effect of long-term TPA treatment on PKC phosphorylation

PKC was immunoprecipitated as described in the legend to Fig. 1. From left to right; immunoprecipitation from control cells, immunoprecipitation from control cells in the presence of competing peptide, high-molecular-mass standards (in descending order 200, 97.4 and 66.2 kDa), and immunoprecipitation from cells treated with 400 nM-TPA for the times (h) indicated on the Figure.

Table 1. Effects of PDGF and EGF on PKC phosphorylation in HFF cells

The effect of PDGF and EGF on [32 P]P_i incorporation into PKC immunoprecipitated from 32 P-labelled HFF cells treated with PDGF (10 ng/ml) or EGF (42 nM) for the times indicated are shown in the Table. The results are means of two or three (PDGF, 20 min) experiments and are expressed as a percentage of control. Maximal stimulation by TPA (400 nM for 60 min) was 400% of control (mean of seven experiments).

Time of treatment (min) . . .	PKC phosphorylation (%)	
	5	20
PDGF	95.5	223
EGF	61.5	45

Surprisingly, EGF treatment of HFF cells caused a decrease in [32 P]P_i incorporation into PKC and this decrease was time-dependent (Table 1). In view of this decrease in response to EGF, it was perhaps not unexpected that foetal-calf serum (10%, for up to 60 min), which is a complex mixture of mitogens, produced only a small increase (20% above controls) in PKC phosphorylation (results not shown); this presumably reflects a balance of effects.

The changes in phosphorylation state of PKC were further assessed by two-dimensional tryptic-peptide mapping of immunoprecipitated PKC from HFF cells after treatment with TPA, EGF or PDGF. Such mapping of PKC immunoprecipitated from HFF cells treated with 400 nM-TPA for 60 min revealed novel tryptic peptides (4 and 5) (Fig. 4) and increased labelling of the major 'basal' tryptic peptide (1) and of the minor basal peptides (2 and 3) (the minor basal peptides 2 and 3 are visible in the original autoradiographs of maps derived from control cells). Two-dimensional tryptic-peptide maps of

Fig. 4. Two-dimensional peptide mapping of PKC immunoprecipitated from 32 P-labelled HFF cells, control or TPA-treated

Immunoprecipitated 32 P-labelled PKC was digested with trypsin and analysed by two-dimensional peptide mapping. The radioactivity (c.p.m.) loaded on the t.l.c. plate was directly proportional to that in the PKC immunoprecipitated from control and treated cells (this was to normalize the recoveries of 32 P-labelled peptide). 32 P-labelled peptides were detected by autoradiography. Directions of electrophoresis (E) and ascending chromatography (C) are as indicated (O, origin). The autoradiograms show the patterns of separated 32 P-labelled tryptic peptides derived from PKC immunoprecipitated from (a) control HFF cells and (b) HFF cells treated with 400 nM-TPA for 60 min, and (c) a schematic representation of the phosphopeptides present in (a) and (b).

Fig. 5. Two-dimensional peptide mapping of PKC immunoprecipitated from 32 P-labelled HFF cells; effects of PDGF or EGF

Tryptic-peptide mapping was carried out as described in the legend to Fig. 4. The autoradiograms show the separation patterns of 32 P-labelled tryptic peptides derived from PKC immunoprecipitated (a) from control HFF cells, (b) from HFF cells treated with PDGF (100 μ g/ml) for 20 min and (c) from HFF cells treated with EGF (42 nM) for 20 min.

Table 2. PKC translocation in HFF cells

PKC activity was measured in partially purified extracts from either control HFF cells or cells treated with TPA (400 nM for 60 min), PDGF (10 ng/ml for 20 min) or EGF (42 nM for 20 min) as described in the Materials and methods section. Activities are expressed as a percentage of the control value in either the supernatant or pellet. Control activities were 37.02 ± 5.44 units/mg for supernatant fractions and 11.75 ± 3.52 units/mg for the pellet fractions. *P* values are from comparison with controls (*n* = 3); **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

	Activity (%)	
	Supernatant	Pellet
Control	100	100
TPA	1.3 ± 0.7 (3)***	250 ± 29 (3)**
PDGF	82 ± 8.0 (4)	147 ± 13.5 (4)*
EGF	107 ± 14.6 (3)	88 ± 14.5 (3)

Fig. 6. Effects of PDGF and TPA treatment on the 80K PKC substrate

Whole-cell lysate was electrophoresed as described in the Materials and methods section. From left to right; whole-cell lysate from control cells and from cells treated with PDGF for the times (min) indicated on the Figure, high-molecular-mass standards (in descending order 97.4 and 66.2 kDa), and whole-cell lysates from cells treated with TPA for the times (min) indicated on the Figure. Arrowheads indicate the 80K protein.

PKC immunoprecipitated from HFF cells treated with EGF (Fig. 5) are similar to those from control HFF cells, although the labelling is less intense. PDGF treatment (Fig. 5), resulted in increased labelling of tryptic peptides compared with control, and this included one of the sites (peptide 3) which is labelled more strongly after treatment with TPA.

In fibroblasts it has been possible to employ the phenomenon of translocation as a means of assessing PKC activation (Halsey *et al.*, 1987). Analysis of the translocation of PKC in HFF cells showed that, although TPA and PDGF caused translocation to the membrane fraction, EGF did not (Table 2). This would be consistent with the increase in the phosphorylation state of PKC in response to TPA and PDGF (see above).

As discussed in the Introduction, another approach to determine PKC activation is the monitoring of the phosphorylation states of defined cellular targets; we have therefore determined the phosphorylation state of the 80K PKC substrate (Rozengurt *et al.*, 1983) in HFF cells. Both PDGF and TPA caused an increase in the phosphorylation state of 80K. Treatment of HFF cells with PDGF resulted in an increase in the phosphorylation state of 80K after 1 min, which was maintained after 5 and 20 min treatment. Treatment with TPA resulted in an increase in the phosphorylation state of 80K after 1 min, which increased further on continued treatment (Fig. 6). These changes in 80K phosphorylation are consistent with PKC activation, again providing a correlation with increased PKC phosphorylation. It should be noted that, although the precise timing of 80K phosphorylation and PKC phosphorylation do not directly correlate (compare Table 2 and Fig. 6), the relative rates of kinase and phosphatase action will markedly influence the rate of attainment of the new steady states of phosphorylation for 80K and PKC.

DISCUSSION

The data presented here demonstrate that, *in vivo*, activators of PKC lead to an increase in the phosphorylation of the kinase itself. This increase is observed with the membrane-permeant (direct) activator TPA as well as with PDGF, which acts in part through receptor-dependent phosphatidylinositol breakdown (the coupling of this process has not been defined). The response to TPA (and PDGF; see Table 1) is time-dependent, reaching a maximum between 20 and 60 min. However, on long-term exposure to TPA (> 1 h) there is a loss of labelled PKC, owing to down-regulation. EGF, which is not thought to lead to PKC activation in fibroblasts, decreases the ^{32}P labelling of PKC in quiescent HFF cells. This response to EGF is also time-dependent (see Table 1). That these changes in PKC phosphorylation provide an indication of activation is suggested by the effects of TPA and PDGF on the translocation of PKC (Table 2) and the phosphorylation of the 80K PKC substrate (Fig. 6).

Peptide mapping of PKC immunoprecipitated from control HFF cells shows that there is one major 'basal' peptide (peptide 1), which does not migrate very far from the origin at pH 3.5. In contrast, PKC from cells treated for an optimum period with TPA show the appearance of four major phosphorylated tryptic peptides (peptides 2–5; Fig. 5). One of these is also observed after PDGF treatment (peptide 3). The original autoradiographs in fact show that in control cells there is weak phosphorylation of both peptides 2 and 3. This pattern of phosphorylation might suggest that the subset of peptides phosphorylated in response to PDGF is due to the relatively transient nature of receptor-linked diacylglycerol production (Hasegawa-Sasaki, 1985), compared with the longer-term effects of TPA, which presumably drives the phosphorylation further. This interpretation is consistent with the observation that PKC- α purified from bovine brain shows a PDGF-like pattern when autophosphorylated at low stoichiometry (F. E. Mitchell,

R. M. Marais & P. J. Parker, unpublished work). Attempts to purify sufficient HFF PKC- α have been unsuccessful, and it remains to be determined whether or not these phosphorylation events are indeed on sites of autophosphorylation *in vitro*.

The effects of EGF on the phosphorylation of PKC are intriguing. The lack of increase in phosphorylation of PKC is clearly consistent with the idea that EGF does not function through this pathway in fibroblasts. The finding that EGF decreases the phosphorylation of PKC suggests that EGF may have a modulatory role on this pathway, and clearly indicates that the phosphorylation state of PKC is regulated in a dynamic fashion. This observation also demonstrates that changes in PKC phosphorylation may also occur through 'cross-talk' with other signal-transduction pathways. Thus the monitoring of PKC phosphorylation provides circumstantial, but not definitive, proof of activation.

The simplest interpretation of these results is that TPA and PDGF lead to an increase in activity of PKC which results in an increase in autophosphorylation. *In vitro*, autophosphorylation has been shown to affect the Ca^{2+} -dependence (Huang *et al.*, 1986b) and the substrate affinity of the kinase (Mochly-Rosen & Koshland, 1987). The changes documented *in vivo*, if due to autophosphorylation, suggest that the autophosphorylation may indeed play a role in modulating the longevity of PKC activation, as suggested previously (Huang *et al.*, 1986b). Whether or not the phosphorylation state of protein kinase C is involved in the regulation of this pathway, it is clear from the data presented here that the extent of phosphorylation can be used as a monitor for the activation of this enzyme, with the caveat that 'cross-talk' may also influence the phosphorylation state (see above). This analysis can be employed in the investigation of growth-factor action. Furthermore, the use of this procedure together with the use of selective antisera to PKC isotypes could allow distinction between activation of different isotypes in cells where different isotypes are co-expressed.

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