Down-regulation of protein kinase C is due to an increased rate of degradation

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The phorbol-ester-induced loss of protein kinase C that has been documented in many cell types appears to be a critical event in the generation of a cellular refractory state. We have investigated here the synthesis and degradation of the protein kinase C polypeptide in order to determine why its steady-state amounts are depleted in response to phorbol esters. These results indicate that depletion is due to an increased rate of degradation, with no change either in mRNA amounts or in rates of polypeptide synthesis.

INTRODUCTION

Phorbol esters have been shown to mimic the effects of diacylglycerol in the activation of protein kinase C *in vitro*. The potent tumour promoters elicit a variety of physiological responses in different target cells, including modulation of ion fluxes (e.g. Seeman *et al.*, 1983; Whiteley *et al.*, 1984) and secretory responses (e.g. Brown *et al.*, 1984; DiVirgilio *et al.*, 1984), changes in gene expression (e.g. Laszlo *et al.*, 1981; Arya *et al.*, 1984), inhibition of growth (reviewed by Gescher, 1985), cell proliferation (e.g. Collins & Rozengurt, 1982a; Bachrach *et al.*, 1985) and differentiation (reviewed by Vandenbark & Nield, 1984). Studies with cultured cells indicate that phorbol esters act through specific receptors to generate these responses (Collins & Rozengurt, 1982b; Nishizuka, 1986; Stabel *et al.*, 1987), and there is now a wealth of evidence to suggest that protein kinase C represents the major high-affinity-binding protein for phorbol esters (see Stabel *et al.*, 1987, and references therein). The implication is that the effects of this class of promoters are transduced, at least in part, through activation of this multi-functional protein kinase.

The cloning of protein kinase C from various species (Parker *et al.*, 1986; Coussens *et al.*, 1986; Knopf *et al.*, 1986; Ono *et al.*, 1986) has shown that there is in fact a family of highly related polypeptides; the functional differences between these species, and therefore the potential consequences of differential expression, remain to be elucidated. The probes developed from this work (cDNA and antibody) now allow detailed analysis of protein kinase C expression in a variety of situations, including phorbol-ester-induced down-regulation.

Early studies showed that chronic exposure of cells to phorbol esters leads to loss or down-regulation of phorbol-ester-binding sites (Solanki *et al.*, 1981; Collins & Rozengurt, 1982a; Phillips & Jaken, 1983). The discovery of the relationship of the phorbol ester 'receptor' to protein kinase C subsequently stimulated several reports, which documented the down-regulation of protein kinase C activity (Rodriguez-Pena & Rozengurt, 1984; Blackshear *et al.*, 1985; Ballester & Rosen,

1985; Stabel *et al.*, 1987). Thus there appears to be a correlation between binding activity, protein kinase C activity, expression of the immunoreactive polypeptide itself and, importantly, phorbol ester responsiveness (see Stabel *et al.*, 1987). The down-regulation of protein kinase C represents a level of control that may play a role in determining cellular responsiveness towards pathological agents such as phorbol esters and also towards hormones and growth factors.

In order to elucidate the mechanism by which phorbol esters induce down-regulation of protein kinase C, we have determined the amounts of protein kinase C mRNA in phorbol-ester-treated cells, and also the rates of synthesis and degradation of the polypeptide itself. These studies show that down-regulation of protein kinase C by phorbol ester is a consequence of an increased rate of degradation of the polypeptide.

EXPERIMENTAL

Materials

Rat glioma cells (328 cells) were provided by Dr. M. Noble, Institute of Neurology, University of London. Dulbecco's modified Eagle's minimal essential medium and foetal-calf serum were supplied by GIBCO, Uxbridge, U.K. All radiochemicals were purchased from Amersham International, Amersham, Bucks., U.K. PMA, Hepes, EDTA, Triton X-100, DL-dithiothreitol, SDS and sodium deoxycholate were from Sigma Chemical Co. Antibodies to protein kinase C were as described elsewhere (Stabel *et al.*, 1987). A cDNA probe to tubulin was kindly provided by Dr. Charles Streuli (Hammersmith Hospital, London).

Incorporation of [³⁵S]methionine

Confluent 328 cells, grown in 50 mm-diam. tissue-culture dishes (Nunc) in Dulbecco's modified Eagle's medium and 10% (v/v) foetal-calf serum with or without 200 nM-PMA, were preincubated for 30 min in medium containing 10% of the usual methionine concentration and 5% dialysed foetal-calf serum. Cells were subsequently labelled by addition of 0.33 mCi of

Abbreviation used: PMA, phorbol 12-myristate 13-acetate.

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[35 S]methionine per dish for 15, 30 or 60 min in the absence or presence of PMA (200 nM). Cells were washed with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate, pH 7.0), lysed by addition of 0.5 ml of 20 mM-Hepes (pH 7.5)/1 mM-EDTA/1.0% Triton X-100/1 mM-dithiothreitol and then centrifuged (2 min at 10000 g). SDS (0.1%) and deoxycholate (1%) were added to the supernatants, which were then cleared with pre-immune serum and fixed *Staphylococcus aureus*. Supernatants were incubated with polyclonal antiserum at room temperature (18 °C) for 45 min, and immunocomplexes were removed on fixed *Staph. aureus* and then washed in lysis buffer with or without 1 M-NaCl. Finally the pellets were boiled in Laemmli (1970) sample buffer and electrophoresed on 8%-polyacrylamide gels in the presence of SDS. Gels were fixed, stained with Coomassie Brilliant Blue (Sigma), dried, and autoradiographed by using Kodak X-ray film (SB-5).

Degradation of [35 S]methionine-labelled protein kinase C

Confluent 328 cells grown on 50 mm-diam. tissue-culture dishes were labelled for 18 h with 0.25 mCi of [35 S]methionine per dish in medium containing 10% of the usual methionine concentration and 5% dialysed foetal-calf serum. Cells were washed with phosphate-buffered saline, then chased in Dulbecco's modified Eagle's medium containing 10% foetal-calf serum in the presence or absence of 200 nM-PMA. Cells were then lysed in boiling sample buffer (Laemmli, 1970), diluted 10-fold with 20 mM-sodium phosphate/5 mM-EDTA/1% Triton X-100/1% deoxycholate, and protein kinase C was immunoprecipitated as described above. Labelled bands were quantified by densitometric scanning of

autoradiographs; multiple exposures of gels were used to ensure linearity.

Determination of mRNA

Total RNA from control and PMA-treated 328 cells was isolated as described previously (Parker *et al.*, 1986); polyadenylated RNA was obtained by selection on oligo(dT)-cellulose (Pharmacia). This mRNA fraction was probed by Northern-blot analysis by using the cDNA clone λ bPKC21 as described elsewhere (Parker *et al.*, 1986); we have established that this cell line exclusively expresses protein kinase C- α mRNA (results not shown). Samples applied to each lane were normalized on the basis of hybridization to a tubulin cDNA probe.

RESULTS

Rates of synthesis of the protein kinase C polypeptide

On pre-treatment of 328 cells with PMA (200 nM for 18 h), there is a severe loss of immunoprecipitable protein kinase C, as judged by the disappearance of the Coomassie-Blue-staining band (Fig. 1a). Thus, as observed in a number of other cell lines, chronic PMA treatment of 328 cells leads to down-regulation of protein kinase C.

In order to determine the rate of synthesis of protein kinase C, cells were labelled with [35 S]methionine for short periods and the newly synthesized protein kinase C was immunoprecipitated. Fig. 1(b) shows that there is no decrease in the rate of incorporation of labelled methionine into protein kinase C in PMA-treated cells relative to controls. A comparison of Figs. 1(a) and 1(b)

Fig. 1. Effect of PMA on steady-state amounts and biosynthesis of protein kinase C

(a) Protein kinase C was immunoprecipitated from 328-cell lysates with the polyclonal antiserum 0442 as described in the Experimental section. Immunocomplexes were boiled in Laemmli sample buffer and subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue to reveal the protein kinase C polypeptide at M_r 79000 (arrow '79k'). Lane A, immunoprecipitation from control cells in the presence of peptide antigen to show specificity of the antibody; lane B, immunoprecipitation from cells treated with PMA (200 nM) for 18 h; lane C, immunoprecipitation from control cells. (b) Confluent 328 cells were grown for 16 h in the presence (lanes C, D, G, H, K, L) or absence (lanes A, B, E, F, I, J) of 200 nM-PMA. Cells were subsequently pulse-labelled with [35 S]methionine as described in the Experimental section, and protein was immunoprecipitated from cell lysates. Arrow indicates protein kinase C. Immunoprecipitation was carried out with antiserum 0442 in the absence (lanes A, C, E, G, I, K) or presence (lanes B, D, F, H, J, L) of competing peptide antigen to demonstrate the specificity of the antiserum. Lanes A-D, 15 min labelling; lanes E-H, 30 min labelling; lanes I-L, 60 min labelling. (c) 328 cells were pulse-labelled with [35 S]methionine for 30 min, followed by a chase in Dulbecco's modified Eagle's medium containing 10% foetal-calf serum. Protein kinase C was immunoprecipitated with the antibody 0442. Lane A, 15 min pulse; lane B, 30 min pulse; lane C, 60 min pulse. Arrows '76k' and '79k' indicate positions of M_r -76000 and -79000 species.

demonstrates that PMA severely decreases steady-state amounts of protein kinase C polypeptide (Fig. 1a) while not affecting the rate of synthesis (Fig. 1b). This emphasizes the distinction between steady-state amounts and rates of synthesis.

It is noteworthy that, during short labelling times (15 min), the major protein kinase C species shows an apparent M_r of 76000, whereas at later times, the predominant species is of M_r 79000 (most clearly shown in Fig. 1c). The faster-migrating species can be chased into the upper band, suggesting some form of post-translational modification (results not shown).

Amounts of mRNA for protein kinase C

In parallel with studying the rates of synthesis of the protein kinase C polypeptide, we have employed a cDNA

Fig. 2. Protein kinase C mRNA expression in control and PMA-treated 328 cells

Polyadenylated RNA was isolated from control and PMA-treated cells as described elsewhere (Parker *et al.*, 1986). The RNA on Northern blots was probed with the protein kinase C clone λ bPKC21 (protein kinase C- α ; see the text), as described in the Experimental section. The positions of 28 S and 18 S rRNA are shown. Lane A, control at zero time; lane B, control after 40 h in culture; lane C, cells treated for 40 h with PMA (200 nM).

Table 1. Quantification of protein kinase C mRNA in PMA-treated 328 cells

Cells were harvested at various times after exposure to PMA (200 nM). Polyadenylated RNA was isolated and hybridized on Northern blots with the protein kinase C cDNA clone λ bPKC21 (Parker *et al.*, 1986). Autoradiographs of the blots were scanned with a Joyce-Loebl densitometer. The same blots were subsequently probed with a cDNA for tubulin and scanned in order to normalize the data. The values show the scan units obtained for protein kinase C mRNA, normalized to the expression of tubulin mRNA at each time point.

Time of PMA exposure (h)	mRNA (scan units)
0	10780
6	11567
9	8156
16	9951
24	8647
40	16332

Fig. 3. Rate of protein kinase C degradation in PMA-treated cells

(a) 328 cells were labelled for 18 h with $[^{35}\text{S}]$ methionine, then chased in medium containing unlabelled methionine for 1 h (lane A), 2 h (lane B), 3 h (lane C) or 5 h (lane D) in the absence (-) or presence (+) of 200 nM-PMA. Protein kinase C was immunoprecipitated from the cell lysates with the antibody 0442. Arrow indicates position of protein kinase C. (b) Autoradiographs of labelled immunoprecipitates as in Fig. 3(a) were scanned with a densitometer to yield peak areas (scan units) in order to determine the half-life of labelled protein kinase C in the presence (▲) or absence (□) of PMA. The data shown are representative for three separate experiments (see the text).

probe to determine whether phorbol ester treatment affects amounts of protein kinase C mRNA. Polyadenylated RNA isolated from 328 cells at different times of PMA treatment was probed on Northern blots with a cloned cDNA (λ bPKC21; Parker *et al.*, 1986) coding for bovine protein kinase C- α [see Coussens *et al.* (1986) for definition of protein kinase C species]. In order to account for differences in the amount of RNA loaded, blots were also hybridized to a tubulin cDNA probe (results not shown). As shown in Fig. 2 and Table 1, there is no alteration in the amounts of the protein kinase C mRNA species during down-regulation of the polypeptide. This is entirely consistent with normal rates of polypeptide synthesis in the presence of PMA (see above). It should also be noted that there is no apparent change in the nature of the major protein kinase C- α mRNA forms.

Degradation of protein kinase C

In order to determine the rate of degradation of protein kinase C, 328 cells were prelabelled overnight with [35 S]methionine and then transferred to normal medium without [35 S]methionine for different lengths of time. The rate of loss of immunoprecipitable [35 S]methionine-labelled protein kinase C was determined for control and PMA-treated cells. Figs. 3(a) and 3(b) show that exposure to PMA leads to an increased rate of loss of [35 S]methionine-labelled protein kinase C. A comparison of the rates of degradation is shown as a semi-log plot (Fig. 3b). Under these conditions, the time required for depletion of 50% of the prelabelled protein kinase C was 2.4 ± 0.1 h (mean $\pm$ S.E.M.; $n = 3$) for PMA-treated cells. By contrast, the loss of 50% of the labelled protein kinase C in control cells required 6.7 ± 0.4 h (mean $\pm$ S.E.M.; $n = 3$).

DISCUSSION

The data presented here clearly demonstrate that the progressive loss of protein kinase C as a result of chronic exposure of 328 cells to phorbol esters is due to an increased rate of degradation, and not to any decrease in the rate of synthesis. Consistent with this observation, we were unable to detect any change in the amounts of mRNA for the kinase. The molecular basis for this increased degradation and down-regulation is not clearly defined. In some superficial respects, this down-regulation does resemble ligand-induced down-regulation of cell-surface receptors, which is in part responsible for homologous desensitization of cells. However, the different compartmentalization of protein kinase C compared with these receptors would implicate other mechanisms involved in the down-regulation of the kinase. There is evidence that the membrane-associated (active) form of protein kinase C is readily susceptible to proteolysis by the Ca^{2+} -activated neutral proteinase (Kajikawa *et al.*, 1983; Melloni *et al.*, 1986). These observations would suggest that the increased rate of degradation in the presence of phorbol esters *in vivo* is likely to be due to membrane-association/activation of the kinase induced by these agents (Kraft & Anderson, 1983).

The phorbol-ester-induced down-regulation of protein kinase C predicts that an increased steady-state concentration of diacylglycerol would also to some degree decrease the steady-state amount of protein kinase C. Such an

increase in diacylglycerol may be associated with transformed cells or proliferating cells relative to their quiescent counterparts; the cells used in the present study cannot be maintained in a quiescent state, and it has not been possible to compare degradation rates under different growth conditions.

The physiological significance of this down-regulation phenomenon is not clear, although it is noteworthy that at least one PMA-resistant cell line is apparently defective in this respect (Solanki *et al.*, 1981). In this context, it will be important to establish the fate of the degraded polypeptide, since *in vitro* the Ca^{2+} -activated neutral proteinase generates a catalytic fragment (M-kinase) that is no longer dependent on Ca^{2+} , phospholipid and phorbol ester (Inoue *et al.*, 1977). The antibody employed in the present studies recognizes the regulatory domain of protein kinase C (Parker *et al.*, 1986), and we have no clear evidence for the appearance of the regulatory domain fragment during down-regulation. Antisera to the catalytic fragment (Parker *et al.*, 1986) can be used to determine the steady-state amounts of this species, although preliminary results (not shown) have not yet identified this fragment *in vivo*.

In defining the rate of synthesis of protein kinase C, we found that the enzyme is initially synthesized as a M_r -76000 species, consistent with the value predicted from the amino acid sequence (Parker *et al.*, 1986). This M_r -76000 form is then converted into a species with an apparent M_r of 79000. This shift in apparent M_r is presumably due to some form of post-translational modification. We have previously identified protein kinase C as a phosphoprotein *in vivo* (Fry *et al.*, 1985), and it is entirely possible that the slower-migrating species represents a phosphorylated form. If this is indeed the case, then it would appear that virtually all the protein kinase C is phosphorylated *in vivo*, since the M_r -76000 species is not detected on equilibrium labelling with [35 S]methionine.

It appears then that the presence of the activator PMA leads to an increased rate of degradation of protein kinase C, with a consequent decrease in the steady-state amounts of the intact polypeptide. The rate at which this steady state is obtained is a function of the relative rates of synthesis and degradation, as is the steady-state concentration that is finally achieved. It may be surmised that individual cell types may behave rather differently in this respect, yielding both different rates and extents of down-regulation of protein kinase C (discussed by Stabel *et al.*, 1987). Because the down-regulation is effected through degradation, and without a shut-down of synthesis, there will inevitably be present a finite concentration of protein kinase C even in the down-regulated state of the cell. Since there is as yet no evidence for other controlling factors (see Stabel *et al.*, 1987), it would appear that the degree of phorbol-ester-induced refractoriness of individual cell lines may vary considerably, as indeed may the phorbol-ester-induced attenuation of specific responses within a given cell type.

Note added in proof (received 5 April 1987)

Woodgett & Hunter (1987) concluded that the down-regulation of protein kinase C in B82L cells was due to increased degradation of the polypeptide, since protein kinase C disappeared with a half-life of 48 min,

whereas there was no change in the rate of protein kinase C synthesis. This conclusion is consistent with the results reported here.

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