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Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines

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Down-regulation of protein kinase C induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was examined in Swiss 3T3, V79, MDBK and C6 cells by Western blotting. Variations in the rate of down-regulation caused by treatment with 100 nM-TPA were observed; TPA treatment for 5 h caused maximal down-regulation in V79 cells, whereas TPA treatment for 10 h or 30 h was needed for maximal down-regulation of protein kinase C in MDBK or Swiss 3T3 cells respectively. The decrease in amount of immunologically detectable protein kinase C was 30% in MDBK cells and 100% in V79 and Swiss 3T3 cells. MDBK and C6 cells could be completely depleted of protein kinase C by treatment with 250 nM-TPA. In C6 cells, after treatment with 500 nM-TPA, an 80% loss of protein kinase C was seen over 10 h. Measurement of the numbers of phorbol-ester-binding sites remaining in each cell line when protein kinase C was maximally down-regulated indicated that in MDBK and Swiss 3T3 cells loss of phorbol-ester-binding sites paralleled loss of protein kinase C, whereas in V79 and C6 cells no such correlation was observed.

INTRODUCTION

Tumour-promoting phorbol esters evoke a wide range of physiological and biochemical responses in cultured cells (reviewed by Blumberg, 1980). These responses are thought to be mediated by interaction of phorbols with a high-affinity receptor (Driedger & Blumberg, 1980), which has been identified as the Ca^{2+}-activated phospholipid-dependent protein kinase termed protein kinase C (Niedel *et al.*, 1983; Ashendell *et al.*, 1983; Kikkawa *et al.*, 1983; Parker *et al.*, 1984; Uchida & Filburn, 1984). Isolation of cDNAs coding for protein kinase C has identified multiple genes encoding different isoenzymes (Parker *et al.*, 1986; Coussens *et al.*, 1986; Knopf *et al.*, 1986; Ohno *et al.*, 1986; Housey *et al.*, 1986), which are expressed in a tissue-specific manner (Knopf *et al.*, 1986).

Activation of protein kinase C by phorbol esters has been associated with translocation of the enzyme from cytosol to membranes (Kraft & Anderson, 1983). At later times, down-regulation of protein kinase C has been detected as a decrease in number of phorbol-ester-binding sites (reviewed by Blumberg *et al.*, 1984), as a decrease in protein kinase C activity (Rodriguez-Pena & Rozengurt, 1984; Blackshear *et al.*, 1985; Chida *et al.*, 1986; Stabel *et al.*, 1987), or as a decrease in amount of the protein kinase C molecule, as indicated by immunoprecipitation or Western blotting (Ballester & Rosen, 1985; Blackshear *et al.*, 1985; Hovis *et al.*, 1986; Stabel *et al.*, 1987; Borner *et al.*, 1988). The studies of phorbol ester binding, carried out on a number of cell types, suggested that there could be cell-type-specific differences in the pattern of down-regulation. Generally phorbol ester binding reached a peak rapidly and then decreased, but the extent and time course of the decrease varied between cell types. In certain cell types, no down-regulation of phorbol-ester-binding sites was observed (Jaken *et al.*, 1981). However, as the experimental conditions used varied quite widely [for example, in some cases subsaturating doses of phorbol ester were used (Solanki *et al.*, 1981; Saloman, 1981)], the significance of these differences is uncertain. In contrast, Western blotting and immunoprecipitation studies, albeit only carried out on a few cell lines, have generally indicated an almost total loss of protein kinase C over a 24 h period (Ballester & Rosen, 1985; Blackshear *et al.*, 1985; Stabel *et al.*, 1987).

Treatment with a single concentration of TPA for a single time period has been used to decrease the amount of protein kinase C in cells (Blackshear *et al.*, 1985; Hovis *et al.*, 1986; Pasti *et al.*, 1986). The experiments described here were carried out to investigate phorbol-ester-induced down-regulation of protein kinase C in various cell lines, by using a series of TPA concentrations and incubation times, and also to examine the correlation between loss of protein kinase C and loss of phorbol-ester-binding sites in these cell lines. We show that different cell lines display a range of sensitivities to TPA-induced down-regulation of protein kinase C, and that loss of phorbol-ester-binding sites does not always correlate with decreased amounts of protein kinase C protein.

MATERIALS AND METHODS

Materials

The cell lines used were: V79 Chinese-hamster lung fibroblasts, clone K, obtained from Dr. R. Newbold, Institute of Cancer Research (ICR), and used between

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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passages 10 and 20; MDBK bovine kidney epithelial cells, obtained from Mr. P. Clapham, ICR, used between passages 5 and 10; Swiss 3T3 mouse fibroblasts, obtained from the ICRF cell production unit, used between passages 3 and 10; and C6 rat glioma cells, obtained from Dr. D. Ventor, ICR, used between passages 25 and 30. V79, MDBK and C6 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum, and Swiss 3T3 cells were grown in Dulbecco's medium containing 5% newborn-calf serum. All cells were grown at 37 °C in a humidified air/CO_2 (9:1) atmosphere.

[^{3}H]PDBu (sp. radioactivity 17.5 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A. TPA, PDBu, phorbol and other chemicals were from Sigma Chemical Co., Poole, Dorset, U.K., and were stored dissolved in dimethyl sulphoxide at −20 °C in the dark. Affinity-purified ^{125}I-Protein A (sp. radioactivity 30 mCi/mg) was from Amersham International, Amersham, Bucks., U.K. Nitrocellulose (0.45 μm pore size) was from Schleicher and Schull, Dassel, Germany. Purified bovine brain protein kinase C, 0442 antiserum and 0442 peptide (Stabel *et al.*, 1987) were generously given by Dr. P. J. Parker, Ludwig Institute for Cancer Research, London W1P 8BT, U.K.

Preparation of cell lysates and Western blotting

Cells were seeded in 6 cm-diameter tissue-culture dishes at 5×10^5 cells/dish and used 2–3 days later, when confluent. After incubation with TPA or phorbol for various lengths of time, cells were rinsed twice in PBS (170 mM-NaCl, 3 mM-KCl, 10 mM-Na_2HPO_4, 6.5 mM-KH_2PO_4) and lysed in 0.3 ml of a buffer consisting of 50 mM-Tris/HCl, pH 7.5, 150 mM-NaCl, 1% Triton X-100, 5 mM-EGTA, 2 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride and 0.01% leupeptin for 15 min on ice. All cellular material was then aspirated from the dish and the supernatant clarified by centrifugation at 12000 *g* for 5 min. Then 20 μl of SDS/PAGE sample buffer (Laemmli, 1970) 5 times the normal concentration and containing 100 mM-dithiothreitol as a reducing agent, was added per 100 μl of supernatant and samples were boiled for 5 min. Small portions of supernatant to which sample buffer had not been added were used for determination of protein concentrations by the Bradford (1976) method, by using the Bio-Rad (Watford, Herts., U.K.) kit with bovine γ-globulin as a standard. Samples containing 150 μg of cellular proteins were resolved by SDS/PAGE on 12.5%-polyacrylamide gels and electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979) at 0.2 A constant current overnight. Non-specific antibody-binding sites were blocked by overnight incubation at 4 °C in PBS containing 0.05% Tween 20 (Batteiger *et al.*, 1982). Blots were then incubated at room temperature with 0442 antiserum diluted 1:600 in PBS on a rotary shaker for 4 h. After three 10 min washes in PBS containing 0.05% Tween 20, blots were incubated with 100000 c.p.m. of ^{125}I-Protein A/ml for 1 h and again washed three times. Autoradiography of blots was carried out at room temperature with Kodak XAR5 X-ray film. Densitometric scanning of autoradiograms was done with a Joyce–Loebl Chromoscan 3 densitometer. Blotted proteins were observed after autoradiography by using Indian Ink staining (Glenney, 1986).

Assay of [^{3}H]PDBu binding to whole cells

Cells were plated in 16 mm-diam. wells at 2×10^5 cells/well, and 100 nM-TPA or -phorbol (or 500 nM-TPA or -phorbol for C6 cells) was added 24 h later, when cells were confluent. Control cells received an equivalent volume of solvent. After a further 30 h incubation, medium was removed and the cell monolayer washed six times over 30 min at 37 °C in serum-free binding medium (Dulbecco's modified Eagle's medium containing 10 mM-Bes and 1 mg of bovine serum albumin/ml) to remove cell-bound phorbol ester (Dunphy *et al.*, 1980). For the binding assay, sextuplicate wells were incubated for $2\frac{1}{2}$ h on ice in 0.2 ml of binding medium containing 60 nM-[^{3}H]PDBu in the presence or absence of 10 μM-PDBu for determination of non-specific binding. Monolayers were then washed in 3×0.5 ml of ice-cold binding medium, lysed in 0.5 ml of 0.1 M-NaOH for 1 h at 37 °C, and 0.2 ml samples were mixed with Packard Fisofluor Scintillation Cocktail and counted for radioactivity in a Beckman LS7500 liquid-scintillation counter. Equivalent non-radioactive lysates were used for measurement of protein concentrations. Specific binding was calculated by subtraction of non-specific binding from total binding and was expressed as mol ($\times 10^{-10}$) of [^{3}H]PDBu bound/100 μg of protein.

RESULTS

Down-regulation of protein kinase C in four cell lines

Lysates were prepared from Swiss 3T3 cells, V79 Chinese-hamster cells, MDBK cells and C6 rat glioma cells after treatment with 100 nM-TPA for times between 30 min and 30 h. As negative controls, untreated cells and cells treated for 30 h with 100 mM-phorbol were included. Portions (150 μg) of cell lysates were resolved on SDS/12.5%-polyacrylamide gels, transferred to nitrocellulose, and probed with antiserum 0442 in the presence or absence of competing peptide. This antiserum was raised against a sequence substantially conserved between the known bovine protein kinase C isoenzymes (Parker *et al.*, 1986; Coussens *et al.*, 1986) and has been demonstrated to react with protein kinase C from various species (Stabel *et al.*, 1987).

In each cell line, reactivity of 0442 antiserum with proteins of apparent molecular masses 80 kDa and 63 kDa was abolished in the presence of peptide (Figs. 1*b*, 1*d*, 1*f* and 1*h*). The 80 kDa protein co-migrated with purified bovine brain protein kinase C (see, e.g., Fig. 1*a*, lanes i and 1). By these two criteria this protein was identified as protein kinase C. The 63 kDa protein has been previously identified as tubulin, the amino acid sequence of which contains a portion homologous to part of the 0442 peptide sequence (Parker *et al.*, 1986; Stabel *et al.*, 1987). Since none of the treatments altered the intensity of the tubulin band, it did not appear that phorbol ester treatment of cells altered their tubulin content. Reactivity of 0442 antiserum with tubulin thus provided a useful internal control on the amount of total cellular protein loaded in each gel track within an individual experiment.

In Swiss 3T3 cells a decrease in the amount of immunologically detectable protein kinase C was apparent after TPA treatment for 2 h, and the amount continued to decrease thereafter, becoming undetectable after 30 h. Phorbol treatment did not alter the amount of

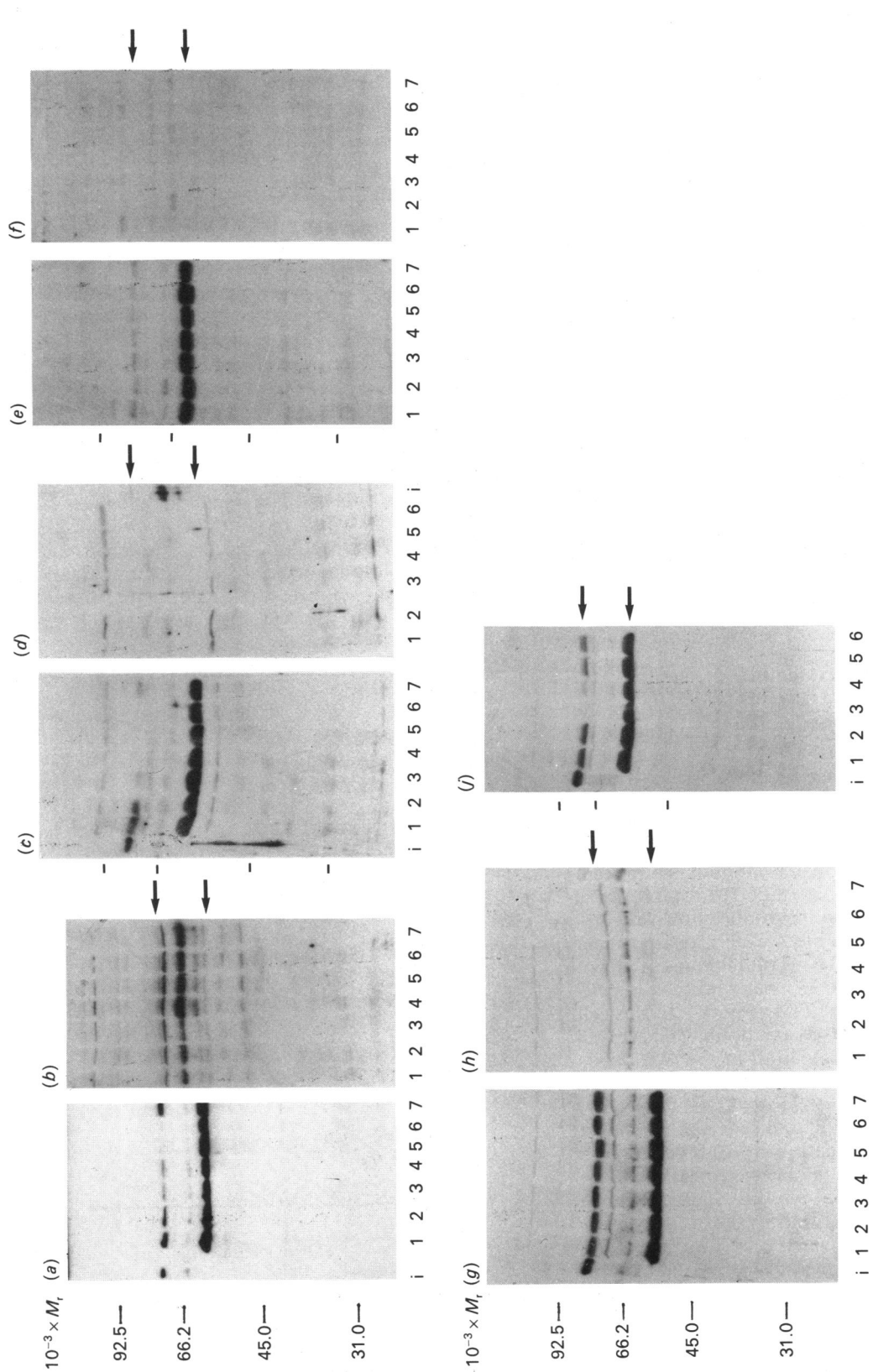

Fig. 1. Down-regulation of protein kinase C in four cell lines, measured by Western blotting

Samples (150 μg) of cell lysates from Swiss 3T3 cells (*a*, *b*), V79 cells (*c*, *d*), MDBK cells (*e*, *f*) and C6 cells (*g*, *h*, *j*) were prepared and Western-blotted as described in the text. Panels (*a*)–(*h*): lane i, 0.5 μg of purified bovine brain protein kinase C; lane 1, lysate of untreated cells; lanes 2–6, lysates from cells treated with 100 nM-TPA for 30 min, 2 h, 5 h, 10 h and 30 h respectively; lane 7, lysate of cells treated for 30 h with 100 nM phorbol. Panel *j*: lane i, 1 μg of purified bovine brain protein kinase C; lane 1, untreated C6 cell lysate; lanes 2–4, lysate of C6 cells treated for 30 h with 100 nM-, 250 nM- or 500 nM-TPA respectively; lanes 5 and 6, lysate treated for 30 h with 100 nM- or 500 nM-phorbol respectively. Panels (*a*), (*c*), (*e*), (*g*), (*j*), blots probed with 0442 antiserum (diluted 1:600); panels (*b*), (*d*), (*f*), (*h*), blots probed with 0442 antiserum (diluted 1:600), preincubated with competing peptide antigen. Arrows indicate proteins not recognized in the presence of peptide. Autoradiographic exposure times: panels (*a*)–(*d*) 5 days; (*e*), (*f*) 4 days; (*g*)–(*j*) 22 h.

Table 1. Down-regulation of protein kinase C in four cell lines

Cells were treated with 100 nM-TPA or -phorbol for the indicated lengths of time, C6 cells also being treated with 500 nM-TPA ($C6_{500}$). Protein kinase C status was examined by Western blotting. Quantitative data on amounts of protein kinase C present (±S.D.; taken from three separate experiments) were obtained by scanning densitometry, samples being normalized on the basis of the intensity of the tubulin band. Lower autoradiographic exposures were used for densitometric purposes than those shown in Fig. 1.

Phorbol ester	Length of treatment (h)	Amount of protein kinase C (% of that in untreated cells)				
		MDBK	Swiss 3T3	V79	$C6_{100}$	$C6_{500}$
TPA	0.5	98±5.0	100±9.8	61±6.7	91±6.4	87±4.2
TPA	2.0	79±4.8	93±5.8	20±5.2	100±4.3	83±2.6
TPA	5.0	66±4.3	57±8.2	0.9±1.5	92±2.1	75±5.8
TPA	10.0	72±6.8	36±9.5	0.7±0.8	90±3.8	17±4.2
TPA	30.0	71±6.4	0	0	100±6.3	16±2.4
Phorbol	30.0	95±3.4	100±10.6	88±16.4	93±12.5	97±5.4

protein kinase C present (Figs. 1*a* and 1*b*). In V79 cells, TPA treatment caused down-regulation of protein kinase C, which also involved a complete loss of detectable protein kinase C. However, the rate of down-regulation was substantially faster than in Swiss 3T3 cells, TPA treatment for 5 h being sufficient to render protein kinase C undetectable (Figs. 1*c* and 1*d*). Although there appeared to be some loss of protein kinase C in response to phorbol treatment for 30 h in this experiment, this result was not reproduced in other experiments.

In contrast, only partial loss of protein kinase C was observed in MDBK cells treated in an identical manner. After 10 h treatment with TPA there was a 30% loss of protein kinase C, and no further loss occurred over the next 20 h. Phorbol treatment did not alter the amount of protein kinase C present (Figs. 1*e* and 1*f*).

The amount of protein kinase C in C6 cells treated with 100 nM-TPA did not alter over a 30 h period (Figs. 1*g* and 1*h*). Comparison of Western blots of lysates of C6 cells with Western blots of the other cell lines indicated that C6 cells contained a greater amount of protein kinase C. To test the possibility that 100 nM-TPA was not an adequate concentration to produce down-regulation in this cell line, Western blots were prepared from C6 cells treated with various doses of TPA and phorbol for 30 h. Loss of protein kinase C was observed in cells treated with 250 nM- or 500 nM-TPA (Fig. 1*j*). In this cell line protein kinase C was resolved as a doublet; there was no evidence for differential regulation of the two bands of the doublet. The time-course experiment was repeated with cells treated with 500 nM-TPA. Loss of protein kinase C was apparent after 5 h, and cells had reached a maximal extent of down-regulation by 10 h, which was maintained over the next 20 h in presence of TPA (Table 1). A dose–response experiment was also carried out on MDBK cells, which were partially resistant to down-regulation of protein kinase C when treated with 100 nM-TPA for 30 h (Fig. 1*e*). MDBK cells were treated with 100 nM-, 250 nM-, 375 nM- or 500 nM-TPA, or 500 nM-phorbol, for 30 h. Cells treated with concentrations of TPA of 250 nM or above became completely depleted of protein kinase C (results not shown). It therefore seemed that lack of down-regulation in C6 and MDBK cells after treatment with 100 nM-TPA was due to failure to stimulate the cells adequately and not to intrinsic unresponsiveness on the part of the cells.

Densitometry was used to quantify the changes in amount of protein kinase C. To ensure that the assay was linear, a Western blot containing different amounts of purified protein kinase C was prepared and used for scanning densitometry. The signal obtained was found to increase linearly with the amount of protein kinase C loaded (Fig. 2). Densitometric measurements were then made from blots loaded with cell lysates. Mean values obtained from several experiments are presented in Table 1.

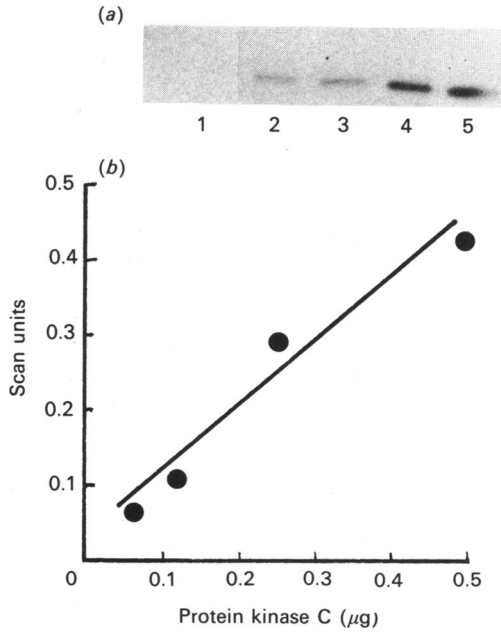

Fig. 2. Quantification of scanning densitometry

(*a*) Autoradiogram obtained from a Western blot probed with antiserum 0442 (diluted 1:600) containing a series of loadings of purified bovine brain protein kinase C: lanes 1–5, loaded with 0.01 μg, 0.06 μg, 0.13 μg, 0.25 μg and 0.50 μg of protein kinase C respectively. (*b*) Plot of scanning-densitometric measurements, in arbitrary units, obtained from such blots.

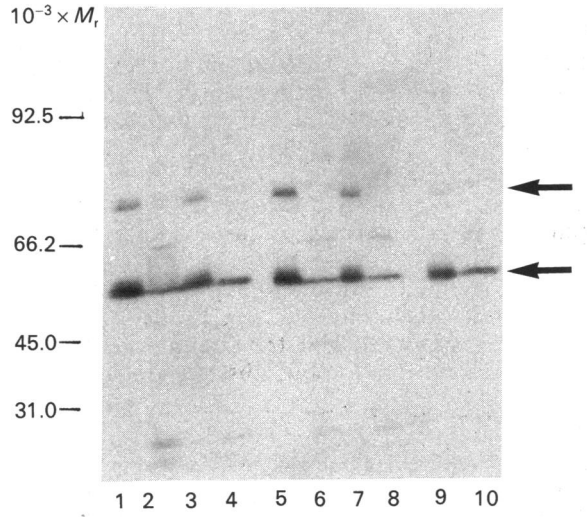

Fig. 3. Efficiency of extraction of protein kinase C as determined by Western blotting

Triton X-100 lysates were prepared from C6 cells, and 150 μg portions of supernatant (lanes 1, 3, 5, 7, 9) or detergent-insoluble material (lanes 2, 4, 6, 8, 10) were analysed by Western blotting and probed with 0442 antiserum as described. Lanes 1, 2, treated with 500 nM-phorbol for 24 h; lanes 3, 4, untreated control; lanes 5, 6, treated with 100 nM-TPA for 24 h; lanes 7, 8, treated with 500 nM-TPA for 30 min; lanes 9, 10, treated with 500 nM-TPA for 24 h. Arrows indicate proteins not recognized in the presence of 0442 peptide. Autoradiographic exposure was 20 h.

Efficiency of extraction of protein kinase C

TPA treatment of cells causes rapid and tight binding of protein kinase C to cell membranes (Kraft & Anderson, 1983). To demonstrate that the lysis buffer used in the above experiments extracted all cellular protein kinase C, lysates were made from C6 cells untreated, treated with 500 nM-TPA for 30 min or 24 h, or treated with 500 nM-phorbol for 24 h. After centrifugation of the lysate, both supernatant and residual insoluble pellet were solubilized in SDS/PAGE sample buffer. SDS/PAGE and Western blotting of both fractions revealed that in untreated cells, phorbol-treated cells and cells treated with TPA for 30 min, all detectable protein kinase C was recovered in the supernatant. After 24 h of TPA treatment there was little protein kinase C in the supernatant, but still no detectable protein kinase C in the detergent-insoluble fraction (Fig. 3). A doublet of proteins which ran below the 31 kDa marker was present in detergent-insoluble fractions from control cells and cells given brief TPA treatment, but was lost from cells treated with 500 nM-TPA for 24 h (Fig. 3, lane 10). Recognition of these proteins by 0442 antiserum was not abolished in the presence of 0442 peptide, indicating that these proteins did not represent fragments of protein kinase C. Thus the results obtained in the down-regulation experiments reflect overall loss of protein kinase C, rather than its translocation into a detergent-insoluble fraction.

Effect of phorbol ester treatment on [^{3}H]PDBu-binding sites

Phorbol-ester-binding sites were examined on cells after treatment for 30 h with 100 nM-phorbol, 100 nM-TPA, or 500 nM-TPA or 500 nM-phorbol in the case of C6 cells. Compared with untreated or phorbol-treated controls, TPA pretreatment decreased specific [^{3}H]PDBu binding to Swiss 3T3 cells by 70% and to V79 cells by 55%. Binding to MDBK cells was decreased by 30%. Treatment of C6 cells with 100 nM-TPA did not decrease [^{3}H]PDBu binding significantly, whereas treatment with 500 nM-TPA caused a 25% decrease in binding (Table 2). Thus, in V79 cells and in C6 cells treated with 500 nM-TPA, the decrease in [^{3}H]PDBu binding was not as great as the decrease in immunologically detectable protein kinase C, which was decreased by 100% and 84% respectively (see Table 1).

DISCUSSION

TPA-induced down-regulation of protein kinase C is caused by an accelerated rate of degradation of the protein kinase C molecule (Woodgett & Hunter, 1987; Young *et al.*, 1987). Degradation may involve proteolysis of membrane-bound protein kinase C by Ca^{2+}-activated neutral proteinase (Kajikawa *et al.*, 1983; Melloni *et al.*, 1985; Chida *et al.*, 1986). Trypsin or calpain treatment of protein kinase C *in vitro* leads to the formation of a 50 kDa fragment with Ca^{2+}- and phosphatidylserine-independent kinase activity (Inoue *et al.*, 1977) and a 32 kDa fragment with Ca^{2+}- and phosphatidylserine-dependent phorbol-ester-binding activity (Huang &

Table 2. Effect of 30 h TPA treatment on [^{3}H]PDBu binding in the four cell lines

[^{3}H]PDBu-binding assays were carried out as described on untreated cells, cells treated with 100 nM- or 500 nM-phorbol for 30 h, or cells treated with 100 nM- or 500 nM-TPA for 30 h. Each value is the mean ± S.D. of sextuplicate determinations; NT, not tested.

	$10^{-10} \times$ [^{3}H]PDBu bound (mol/100 μg of protein)			
Treatment Cell line...	MDBK	Swiss 3T3	V79	C6
Untreated	0.91 ± 0.06	1.05 ± 0.08	1.00 ± 0.15	2.28 ± 0.04
100 nM-phorbol, 30 h	0.90 ± 0.08	1.03 ± 0.04	0.98 ± 0.01	2.23 ± 0.03
100 nM-TPA, 30 h	0.63 ± 0.02	0.21 ± 0.03	0.45 ± 0.02	2.18 ± 0.05
500 nM-phorbol, 30 h	NT	NT	NT	2.24 ± 0.08
500 nM-TPA, 30 h	NT	NT	NT	1.66 ± 0.05

Huang, 1986; Lee & Bell, 1986; Hoshijima *et al.*, 1986). Activation of platelet protein kinase C results in the production of similar fragments (Hoshijima *et al.*, 1986). It is not yet known whether similar processes occur in other cell types.

In the present study, TPA-induced down-regulation of protein kinase C was examined in four cell lines, and differences in the rate and sensitivity of down-regulation were observed. Swiss 3T3 cells, as previously reported (Stabel *et al.*, 1987), completely lost immunoreactive protein kinase C over a 30 h period, whereas in C6, V79 and MDBK cells down-regulation was complete by 10 h. TPA at 100 nM caused complete down-regulation in Swiss 3T3 and V79 cells, whereas treatment with at least 250 nM-TPA was required in MDBK and C6 cells. The concentration needed did not correlate with the abundance of protein kinase C in a particular cell line, since MDBK cells, which contained relatively less protein kinase C, were less TPA-sensitive than Swiss 3T3 or V79 cells. The different patterns of down-regulation observed may reflect expression of protein kinase C isoenzymes in different ratios in the four cells lines, given that each isoenzyme may have a different sensitivity to TPA activation, or be down-regulated at a different rate. For example, the complete lack of down-regulation in C6 cells treated with 100 nM-TPA could be explained if C6 cells contain large amounts of a relatively low-affinity isoenzyme. Variations between cell lines in the amount of Ca^{2+}-activated neutral proteinase present, or in susceptibility of isoenzymes to cleavage by this enzyme, would also lead to differences in the down-regulation rates. Antibodies capable of distinguishing between protein kinase C isoenzymes and of recognizing the 50 kDa, proteolysed, form of protein kinase C are needed to investigate these possibilities further.

Loss of phorbol-ester-binding sites paralleled loss of protein kinase C in Swiss 3T3 and MDBK cells, but was substantially less in C6 and V79 cells. This could be due to the different methods of analysis which examine intact cells or cell lysates, if, for some reason, the plasma membranes of C6 and V79 cells are relatively less permeable to phorbol esters. However, estimates of binding-site numbers on C6 cells obtained by Scatchard analysis (results not shown) corresponded reasonably well to estimates made by comparison of signal intensity obtained on Western blots of C6-cell lysates with that obtained from a known amount of purified protein kinase C.

An alternative explanation is that limited proteolysis of activated protein kinase C releases a phorbol-ester-binding fragment, as described *in vitro* and in platelets (Huang & Huang, 1986; Lee & Bell, 1986; Hoshijima *et al.*, 1986), and this fragment is retained for longer in C6 and V79 cells than in Swiss 3T3 or MDBK cells. However, 0442 antiserum is known to detect the 32 kDa fragment produced on tryptic cleavage of purified brain protein kinase C (Parker *et al.*, 1986), yet no such fragment was detected on the Western blots. It has previously been noted that this antiserum did not detect such a fragment of protein kinase C in immunoprecipitates of a glioma-cell line after TPA treatment (Young *et al.*, 1987). Possibly the antiserum is insufficiently sensitive to detect this fragment in cell lysates. However, while the present paper was in preparation, it was reported that antiserum 0442 detected proteins of apparent molecular masses 31–35 kDa after TPA-induced down-regulation of protein kinase C in mouse skin (Fournier & Murray, 1987).

In summary, these experiments indicate that the four cell lines examined differ in their sensitivity to and rate of TPA-induced down-regulation of protein kinase C. The parameters of down-regulation should therefore be established for any new cell type studied, particularly if it is wished to use TPA down-regulation to produce a population of cells deficient in protein kinase C (Blackshear *et al.*, 1985; Hovis *et al.*, 1986; Pasti *et al.*, 1986). Further, the results suggest that phorbol-ester-binding sites are not necessarily lost in parallel with protein kinase C in response to TPA treatment. The biochemical processes responsible for this observation remain to be elucidated.

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