Activation by phorbol esters of protein kinase C in MCF-7 human breast cancer cells

Marc Issandou, Francis Bayard and Jean-Marie Darbon*

Inserm U 168, Department of Endocrinology, Chu Rangueil, Université Paul Sabatier, 31054 Toulouse Cedex, France

Received 25 February 1986

Exposure of MCF-7 human breast cancer cells to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) leads to the inhibition of cell proliferation. We investigate here the short-term effects of TPA on subcellular distribution of protein kinase C, and on protein phosphorylation in cultured MCF-7 cells. We report a rapid and dramatic decrease in cytosolic protein kinase C activity after TPA treatment. Only 30% of the enzymatic activity lost in the cytosol was recovered in the particulate fraction. These data suggest that subcellular translocation of protein kinase C is accompanied by a rapid down-regulation of the enzyme (70%). Furthermore, TPA and other protein kinase C activators rapidly induce the phosphorylation of a 28 kDa protein in intact MCF-7 cells. Phorbol esters devoid of tumor-promoting activity are ineffective both for inducing these early biochemical events and for inhibiting cell proliferation.

Protein kinase C *Phorbol ester* *Diacylglycerol* *Protein phosphorylation*

1. INTRODUCTION

Tumor-promoting phorbol esters induce various and often opposite biological and biochemical effects on cells in culture. Depending on the cell system, they can either stimulate proliferation and inhibit differentiation [1,2], or induce growth arrest and cell differentiation [3,4]. The exposure of MCF-7 human breast cancer cells to TPA results in a dose-dependent inhibition of cell proliferation [5–7]. Although the mechanism of action of the phorbol esters is not clearly understood as yet, they are thought to act through the calcium- and phospholipid-dependent protein kinase (protein kinase C), which has been proposed as their apo-receptor [8]. This enzyme is activated by diacylglycerol produced by the breakdown of membrane inositol phospholipids. Phorbol esters can substitute for diacylglycerol and activate the protein kinase C directly [9]. We have investigated the short-term effects of TPA on protein kinase C activity in MCF-7 cells and found that TPA dramatically and rapidly decreased the cytosolic protein kinase C content, with a lack of complete recovery of the enzyme in the particulate fraction. We also report the effects of protein kinase C activators TPA, OAG and phospholipase C on protein phosphorylation in intact MCF-7 cells.

* To whom correspondence should be addressed

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBU, phorbol 12,13-dibutyrate; DMSO, dimethyl sulfoxide; OAG, 1-oleoyl-2-acetylglycerol; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Chemicals*

H1 histone type IIIS, 8-Br-cAMP, TPA, PDBU and phospholipase C (from *Clostridium welchii/perfringens*) were obtained from Sigma. [^{32}P]Phosphoric acid and [γ-^{32}P]ATP (0.5–3 Ci/mmol) were purchased from Amersham. DEAE cellulose (DE52) was from Whatman. Acrylamide and bisacrylamide were obtained from Eastman Kodak. X-ray films (X-Omat AR) were from

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

Kodak. All other chemicals were from Merck and OAG was a gift from Dr H. Chap (Inserm U 101, Toulouse).

2.2. *Methods*

2.2.1. Cell cultures

MCF-7 cells were grown at 37°C in RPMI 1640 medium, pH 7.3, supplemented with 2 g/l of sodium bicarbonate, 2 mM L-glutamine, 1 μM insulin and 5% fetal calf serum.

2.2.2. Subcellular fractionation

Subconfluent cells were scraped from the culture dishes into phosphate-buffered saline (PBS), then homogenized in 20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 50 μg/ml PMSF, 5 mM β-mercaptoethanol (buffer A). The cell lysate was centrifuged for 1 h at 105000 × g. The supernatant was used as the cytosolic fraction and the pellet was resuspended in buffer A, containing 0.5% Triton X-100, stored at 4°C for 45 min and centrifuged for 1 h at 105000 × g. The supernatant recovered was used as the 0.5% Triton extract of the particulate fraction. Protein concentrations were determined according to Lowry et al. [10], taking into account the presence of Triton.

2.2.3. DEAE cellulose chromatography

Cytosol or 0.5% Triton extract of particulate fraction obtained from 60 × 10^6 cells was applied to a DEAE cellulose column (DE52, 0.7 × 5 cm), equilibrated with 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM EGTA, 50 μg/ml PMSF and 5 mM β-mercaptoethanol (buffer B). After washing with 30 ml of buffer B, containing 10 μM cAMP, a linear gradient of NaCl (0–0.35 M) in the same buffer was used to eluate proteins from the column. An aliquot of each collected fraction (1 ml) was immediately assayed for protein kinase activity.

2.2.4. Protein kinase assay

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone. The standard assay mixture (200 μl) contained 20 mM Tris-HCl, pH 7.4, 40 μg histones, 10 μM ATP (550 cpm/pmol), 5 mM $MgCl_2$, 40 μl sample and either 0.5 mM EGTA or 0.5 mM $CaCl_2$, 16 μg phosphatidylserine and 0.6 μg 1,2-diolein. After incubation for 5 min at 30°C, 10% trichloroacetic acid was added to stop the reaction. The protein precipitate was dissolved in 1 N NaOH and the incorporation of ^{32}P was measured by scintillation counting in Pico-fluor 15 (Packard). Enzymatic activity was expressed as pmol ^{32}P incorporated into histone per min.

2.2.5. Protein phosphorylation

Subconfluent cultures (1 × 10^6 cells/dish) were washed twice in a phosphate-free Krebs-Ringer buffer, pH 7.2, containing 20 mM Hepes, 0.1% BSA and 0.2% glucose, then incubated for 2 h in 1 ml of the same buffer containing 50 μCi [^{32}P]phosphoric acid. Stimuli were added for a further 30 min period. Cells were washed twice with cold PBS and 10% trichloroacetic acid was added. Trichloroacetic acid-precipitated proteins were dissolved in 100 μl of electrophoresis sample buffer, containing 0.06 M Tris-HCl, pH 6.7, 2% SDS, 8% glycerol, 2% β-mercaptoethanol, 0.005% bromophenol blue. Samples were then boiled for 5 min at 100°C. Proteins were fractionated by electrophoresis on 4.5 and 10% (w/v) SDS-polyacrylamide slab gels as described by Laemmli [11]. After protein fixation by cold trichloroacetic acid and Coomassie blue staining [12], the gels were dried, then exposed to Kodak X-Omat AR films for 24–48 h. The autoradiographs were scanned by densitometry, and the ^{32}P incorporated into the proteins was evaluated by measuring the respective peak areas.

3. RESULTS AND DISCUSSION

When histone kinase was measured in crude cytosols from MCF-7 cells, no significant protein kinase C activity could be detected (fig.1). Identical results were obtained when whole homogenates were used instead of cytosolic fractions (not shown). This inability to measure protein kinase C activity in crude cell extracts has been already reported in other systems [13,14], and attributed to the presence of specific inhibitors [15] or phosphatase [16] in the extracts.

Fig.2A shows the elution profile of protein kinase activity when cytosol from MCF-7 cells was analysed by DEAE cellulose chromatography.

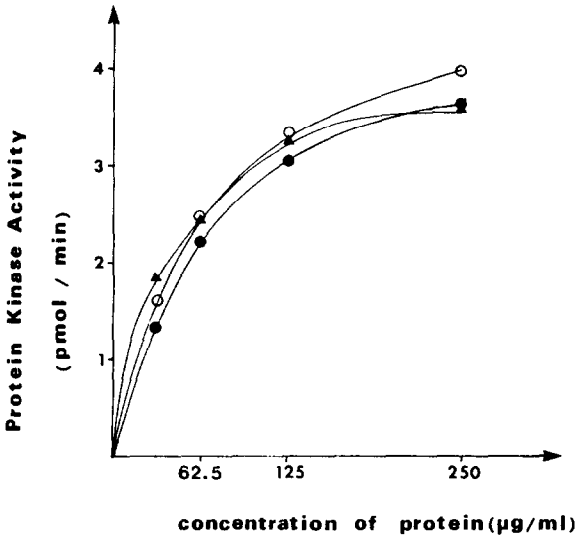

Fig.1. Protein kinase activity in crude cytosolic extracts from MCF-7 cells. The 105000 × *g* supernatant was prepared from subconfluent cells. Protein kinase activity was measured as a function of cytosol dilution, in the presence of 0.5 mM EGTA (○), 0.5 mM Ca^{2+} (●), and 0.5 mM Ca^{2+}, 16 µg phosphatidylserine and 0.6 µg diolein (▲).

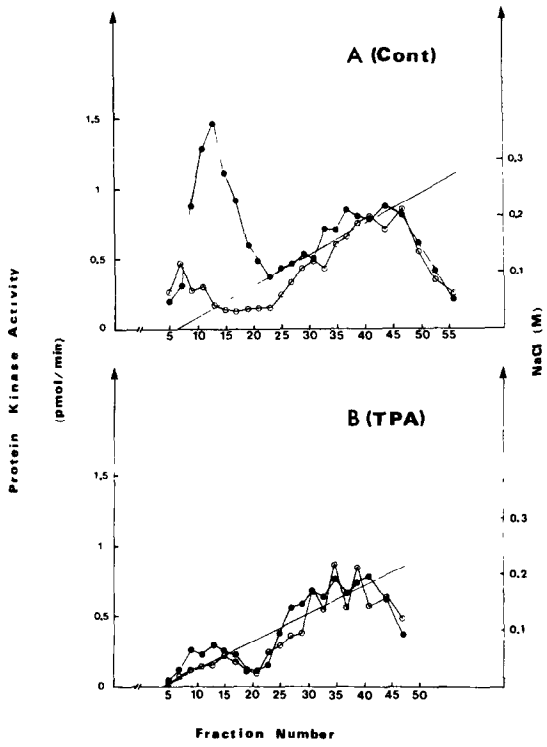

Fig.2. DEAE cellulose chromatography of MCF-7 cytosol. The 105000 × *g* supernatants (10 mg of protein each) from control cells (Cont, A) or from cells treated for 10 min with 100 ng/ml TPA (B) were fractionated on DEAE cellulose. Proteins were eluted with a linear gradient of NaCl (0–0.35 M). Protein kinase activity was measured in the presence of 0.5 mM EGTA (○) and in the presence of 0.5 mM Ca^{2+}, 16 µg phosphatidylserine and 0.6 µg diolein (●). TPA was dissolved in DMSO. The control contained the same final solvent concentration (0.05%).

Protein kinase C activity was eluted as a single peak at 0.04 M NaCl, while a broader peak of calcium- and phospholipid- (Ca, PL-)independent protein kinase activity was eluted between 0.13 and 0.2 M NaCl. This latter protein kinase activity probably corresponds to the protein kinase M, already described in other systems [17,18].

Fig.3 shows that TPA can substitute for diacylglycerol in activating the protein kinase C eluted from DEAE cellulose. These results confirm those obtained with calcium- and phospholipid-dependent protein kinases from other cell types or tissues.

Fig.2B shows that a previous treatment of MCF-7 cells with 100 ng/ml TPA for 10 min leads to a dramatic decrease (about 90%) of the cytosolic protein kinase C activity, while the Ca, PL-independent protein kinase activity was almost unaffected. The inactive tumor promoter 4-*O*-methyl-TPA was unable to induce this effect (not shown).

The principal finding of this work is illustrated in fig.4. In this experiment, protein kinase C activity was measured both in the cytosolic and in the 0.5% Triton extract of the particulate fraction, after DEAE cellulose chromatography. (As in the cytosolic fraction, no protein kinase C activity was measurable in the 0.5% Triton extract from particulate fraction before fractionation on DEAE cellulose.) Two major facts may be observed:

(i) The particulate fraction from non-treated cells contains an important bulk of protein kinase C activity (approx. 40% of the total amount of protein kinase C in MCF-7 cells). This particulate protein kinase C did not appear

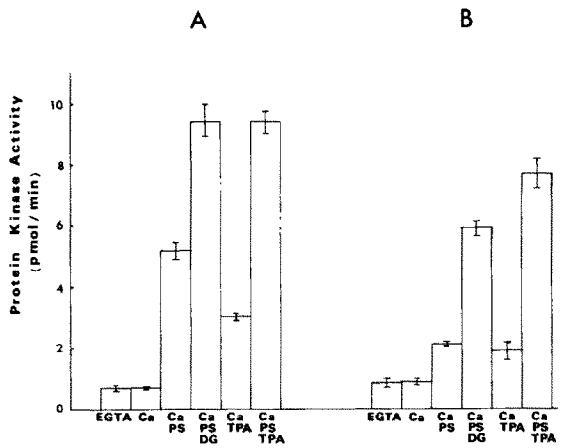

Fig.3. Diacylglycerol- and TPA-dependence of protein kinase C from MCF-7 cells. Cytosolic (A) or 0.5% Triton extract of particulate fraction (B) were fractionated separately on DEAE cellulose and the respective peaks of protein kinase C were studied. Protein kinase activity was measured in the presence of the indicated compounds. The concentrations used were those of the standard assay. TPA was added at 10 ng/ml in DMSO (0.05% final concentration).

basically different from the cytosolic enzyme. The same pattern of elution was obtained for both enzymatic activities (a major peak eluted at 0.04 M NaCl), following DEAE cellulose chromatography. Both enzymes show a similar behavior with regard to diacylglycerol- or TPA-dependence (fig.3).

(ii) The dramatic decrease of the cytosolic protein kinase C content, induced by TPA is accompanied by a very partial increase in the amount of particulate enzyme. Only 30% of the protein kinase C, lost at the cytosolic level, was recovered in the particulate fraction. Moreover, a part of the enzyme appeared more tightly bound to the resin and was eluted between 0.1 and 0.2 M NaCl, which would suggest a modification of the physicochemical properties of the enzyme after TPA action.

The incomplete recovery of the cytosolic enzyme at the particulate level cannot be explained by an inhibition of the enzyme in this fraction as protein kinase activities were measured after fractionation on DEAE cellulose. It can be objected that Triton X-100 induces a certain degradation of the enzyme in the particulate fraction during the extraction step. We feel that it is not the case as we have demonstrated that previous exposure of cytosolic enzyme to 0.5% Triton before DEAE cellulose chromatography did not modify the enzymatic activity (not shown). Furthermore, the enzyme recovery was only poorly improved when the particulate protein kinase C was extracted with Nonidet P-40 instead of Triton X-100. Therefore, our results plead in favor of a rapid down-regulation of the translocated enzyme upon TPA action. Such a phenomenon has been reported very recently in chronic lymphocytic leukemia cells [19] and distinguished from the slow down-regulation induced by long-time exposure of 3T3 cells to TPA [20]. We have also observed an almost complete disappearance of protein kinase C activity in MCF-7 cells after 24 h exposure to TPA (J.M. Darbon et al., in preparation). In any case, our results differ from those reported in parietal yolk sacs (PYS-2) cells, where the enzyme recovery at the membrane level was almost complete [21].

Fig.5 shows the protein phosphorylation pattern obtained when cultured MCF-7 cells were stimulated for 30 min, by different activators of protein kinase C. Phorbol esters, TPA and PDBU, phospholipase C (known to induce the formation of diacylglycerol at the cell membrane level) and synthetic diacylglycerol OAG markedly increase the phosphorylation of at least one protein of apparent M_r 28000. 100 ng/ml TPA, 100 ng/ml PDBU, 0.25 U/ml phospholipase C and 25 μg/ml OAG respectively enhance the phosphorylation of this protein by 600%, 500%, 375% and 300%. Moreover, there is a clear lack of synergism between OAG and TPA, or OAG and phospholipase C, suggesting that these different compounds act, as expected, by the same mechanism, i.e. activation of protein kinase C. 8-Br-cAMP did not induce this specific protein phosphorylation, indicating that the 28 kDa protein is not a substrate for the cAMP-dependent protein kinase. The inactive tumor promoter 4α-phorbol was as ineffective on protein phosphorylation as 4-*O*-methyl-TPA was without effect on protein kinase C translocation. In the same way, while active phorbol esters TPA and PDBU induced growth arrest of MCF-7

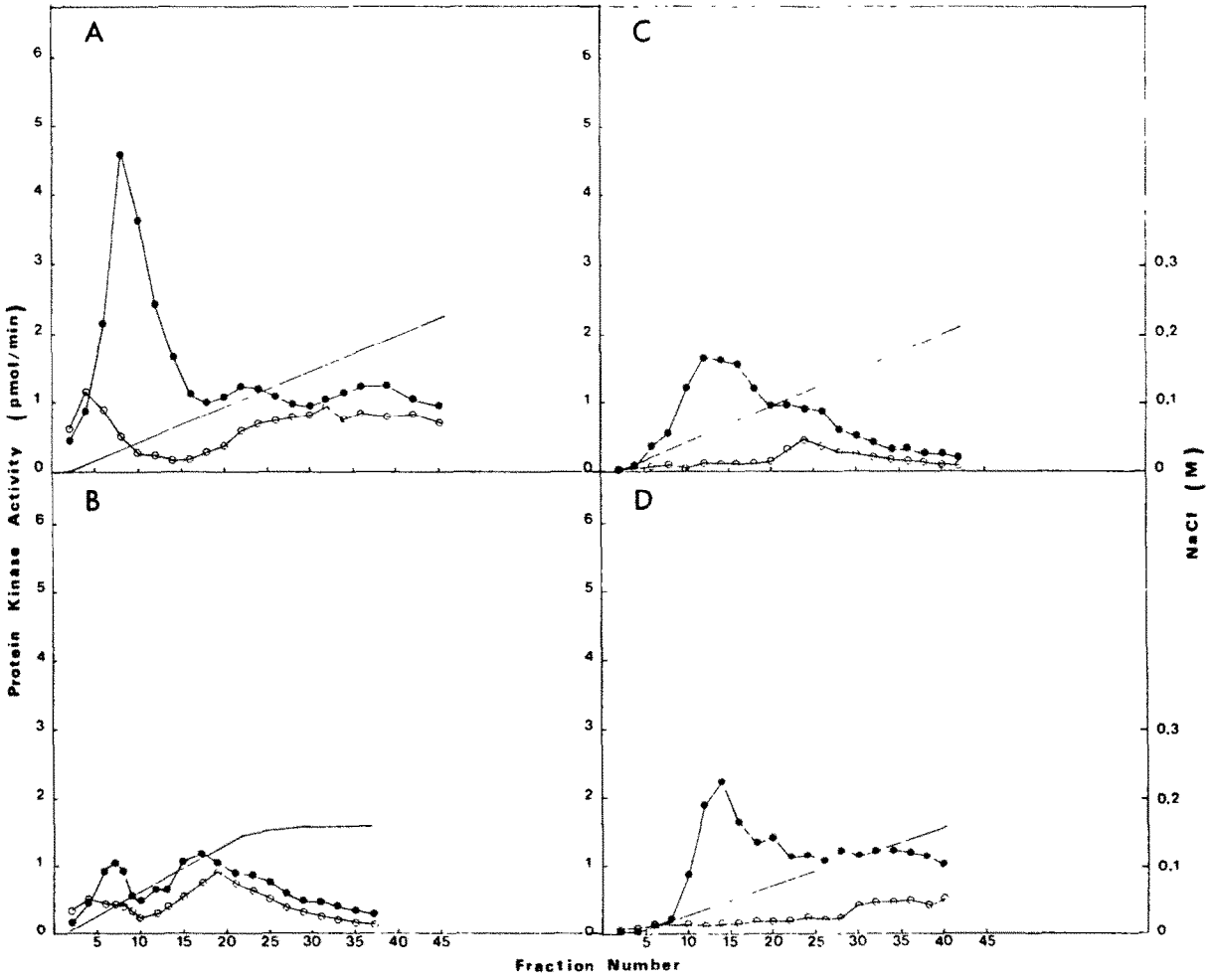

Fig.4. Effect of TPA on subcellular distribution of protein kinase C in MCF-7 cells. The cytosolic fractions (A,B) and the 0.5% Triton extracts of particulate fractions (C,D) were prepared from 60×10^6 cells treated with 0.05% DMSO (control, panels A,C) and from 60×10^6 cells treated with 100 ng/ml TPA for 10 min (panels B,D), then submitted to DEAE cellulose fractionation. Protein kinase activity was measured in the presence of 0.5 mM EGTA (○) or 0.5 mM calcium, 16 μg phosphatidylserine and 0.6 μg diolein (●).

cells, the phorbol derivatives devoid of tumor promoting activity were without effect on cell proliferation [7].

In conclusion we have demonstrated that the treatment of MCF-7 cells with TPA leads to a rapid and dramatic decrease of cytosolic protein kinase C. Only 30% of the activity lost at the cytosolic level seemed translocated to the particulate fraction, while 70% were apparently down-regulated. Moreover, we have shown that TPA and other protein kinase C activators rapidly induced the phosphorylation of a 28 kDa protein in intact MCF-7 cells. These early biochemical events may be related to the biological effect of TPA on MCF-7 cells, i.e. the inhibition of cell proliferation.

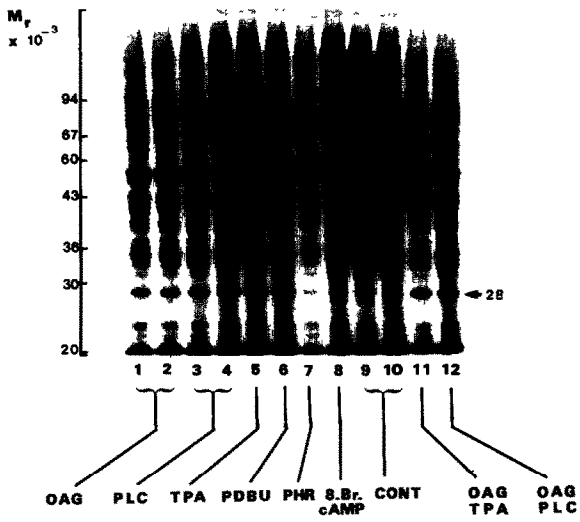

Fig.5. Effects of protein kinase C activators on the protein phosphorylation pattern in intact MCF-7 cells. Subconfluent cells were incubated with 50 μCi [^{32}P]phosphoric acid, in the absence (9) or presence of 0.05% DMSO (10); 12.5 (1) or 25 μg/ml OAG (2); 0.125 (3) or 0.25 U/ml phospholipase C (4); 100 ng/ml TPA (5); 100 ng/ml PDBU (6); 100 ng/ml 4α-phorbol (7); 5 mM 8-Br-cAMP (8); 12.5 μg/ml OAG plus 100 ng/ml TPA (11); 12.5 μg/ml OAG plus 0.125 U/ml phospholipase C (12). Phorbol esters and OAG were dissolved in DMSO (final concentration of 0.05%). Trichloroacetic acid-precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and radioactive proteins visualized by autoradiography. M_r values were evaluated by the use of standard protein markers.

ACKNOWLEDGEMENTS

This study was supported by the Institut National de la Recherche Médicale. The authors thank Mrs F. Delassus for her skillful technical assistance and Mrs M. Larribe for the typing of the manuscript.

REFERENCES

[1] Collins, M.K.M. and Rozengurt, E. (1982) J. Cell. Physiol. 112, 42–50.
[2] Bachrach, L.K., Eggo, M.C., Mak, W.W. and Burrow, G.N. (1985) Endocrinology 116, 1603–1609.
[3] Huberman, E. and Callaham, M.F. (1979) Proc. Natl. Acad. Sci. USA 76, 1293–1297.
[4] Rosoff, P.M., Stein, L.F. and Cantley, L.C. (1984) J. Biol. Chem. 259, 7056–7060.
[5] Osborne, C.K., Hamilton, B., Nover, M. and Ziegler, J. (1981) J. Clin. Invest. 67, 943–951.
[6] McKay, I., Collins, M.K.M., Taylor-Papadimitriou, J. and Rozengurt, E. (1983) Exp. Cell Res. 145, 245–254.
[7] Darbon, J.M., Valette, A. and Bayard, F. (1986) Biochem. Pharmacol., in press.
[8] Leach, K.L., James, M.L. and Blumberg, P.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4208–4212.
[9] Castagna, M., Takai, Y., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Darbon, J.M., Ursely, J., Mangue, N. and Leymarie, P. (1980) FEBS Lett. 113, 120–124.
[13] Kraft, A.S. and Anderson, W.B. (1983) J. Biol. Chem. 258, 9178–9183.
[14] McCaffrey, P.G., Friedman, B. and Rosner, M.R. (1984) J. Biol. Chem. 259, 12502–12507.
[15] Schwantke, N. and Le Peuch, J. (1984) FEBS Lett. 177, 36–40.
[16] Sahyoun, N., Levine, H., McConnell, R., Bronson, D. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. USA 80, 6760–6764.
[17] Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
[18] Vilgrain, I., Cochet, C. and Chambaz, E.M. (1984) J. Biol. Chem. 259, 3403–3406.
[19] Wickremashinghe, R.G., Piga, A., Campana, D., Yaxley, J.C. and Hoffbrand, A.V. (1985) FEBS Lett. 190, 50–54.
[20] Rodriguez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053–1059.
[21] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.