Abnormal behavior of protein kinase C in the human myeloma cell line, RPMI 8226

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Protein kinase C activity of the human myeloma cell line, RPMI 8226, was studied after prepurification on DEAE-cellulose. The total protein kinase activity, eluted at 0.12 M NaCl, was 493 nmol/min/10^{10} cells, but 38% was associated with membranes. The lipid dependence of cytosolic and membrane activities was only 52% and 21%, respectively. This activity increased with time, to as much as 200% for the membrane fraction after 7 days, whereas lipid dependence and the PDBu binding properties were lost. This modified activity was not due to the extinction of a copurifying endogenous inhibitor nor to classical PKC proteolysis. TPA-treatment of these cels is accompanied by a rapid, selective and complete loss of lipid-dependent activity of the cytosol, thus benefiting co-migrating lipid independent activity, with no membrane fraction recovery or PKM formation.

Myeloma cell line; RPMI 8226; B lymphocyte; Protein kinase C

1. INTRODUCTION

PKC, a closely related protein kinase family, is one of the major regulatory enzymes implicated in the transmission of extracellular signals into the cells. It plays pivotal roles in cell proliferation and differentiation, although the key target substrates have not yet been identified. Its identification as the major cellular receptor for tumor promoting phorbol esters also points to its involvement in tumorigenesis, and many efforts have currently been directed towards interpreting this role [1-3]. Cells altered by a variety of oncogenes show modifications which lead to persistent PKC activation [3-5]. Cellular overexpression of normal PKC species [6,7] results in altered growth regulation and induces oncogenicity as observed by mutation [8] or phorbol-ester treatment [1-3]. We recently showed that PKC function was indispensible for triggering L_2C guinea pig leukemic B lymphocyte proliferation [9]. In this study, we observed quite abnormal behavior of PKC activity in the human myeloma cell line RPMI 8226.

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Abbreviations: DAG, diacylglycerol; E 64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

2. MATERIALS AND METHODS

2.1. *Chemicals and biological materials*

[γ-^{32}P]ATP and [^{3}H]PDBu were from Amersham and NEN respectively. L-α-phosphatidylserine, 1,2-dioleoyl-sn-glycerol and histone H1 type IIIS were from Sigma. The Epstein-Barr virus-negative RPMI 8226 cell line has lymphoplasmocyte cells phenotype and is a model of myeloma tumor stem cells [10]. Cells were grown in RPMI 1640 (Boehringer Mannheim) supplemented with 1 mM glutamine, 50 μM β-mercaptoethanol and 10% fetal calf serum. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator.

2.2. *Preparation of cytosolic and particulate fractions*

In standard conditions, 1-2 10^8 cells were suspended in 10 ml of buffer A at 4°C (20 mM Tris-HCl, pH 7.5, 300 mM sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml of leupeptin and E64). Unbroken cells were removed by centrifugation at 1000 × *g* and the homogenate was centrifuged for 30 min at 100000 × *g*. The supernatant (cytosolic fraction) was kept at 4°C whereas the pellet (membrane fraction) was resuspended for 1 h in 10 ml of buffer A containing 0.5% Triton X-100. The extracts were then adsorbed onto 5 ml of DE-52 ion exchange cellulose (Whatman) for 30 min before installation of the resin in columns connected to an FPLC apparatus (Pharmacia), and equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, 2 mM EDTA and EGTA, 1 mM dithiothreitol). Columns were washed through with 4 volumes of the equilibration buffer before elution as indicated in the text. Aliquots of each fraction were then assayed for PKC (30 μl) or PDBu binding (20 μl). In some experiments, protein kinase C from rat brain was obtained after partial purification on DE-52 [11]. Its activity was more than 90% dependent on lipids.

2.3. *Determination of protein kinase C*

Assays were performed using 500 μg/ml of histone H1, type III S as the substrate and 50 μM [γ-^{32}P]ATP (100-250 dpm/pmol) in 96-multiwell dishes [11]. When indicated, activities were calculated from the difference in ^{32}P incorporated into histone in the presence and absence of added phosphatidylserine, diolein, and Ca^{2+} (replaced

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by 0.5 mM EGTA). Each unit of protein kinase activity corresponds to 1 nmol of phosphate transferred to histone H1/min at 25°C. [^{3}H]PDBu binding was measured with mixed micelles of Triton X-100 containing 0.3% Triton X-100 and 0.6 mg/ml of phosphatidylserine and 50 nM [^{3}H]PDBU (20.8 Ci/mmol). Non-specific binding was determined by adding a 7.5 μM excess of unlabelled PDBu. The bound [^{3}H]PDBu was separated from the free ligand after absorption ot DEAE-Sephacel [11].

3. RESULTS

Prior to the experiment, cells were maintained for 3 days in complete medium, and then the medium was removed and cells incubated for 24 h in a fresh medium at a cell density of 8–10 10^6 cells/ml. This refreshment procedure seems to provide the best experimental conditions for comparing protein kinase activities.

PKC activity of cytosol and particulate fractions of RPMI 8226 cells was determined after prepurification on DEAE-cellulose columns. The elution showed 2 peaks of kinase activity in the cytosol (Fig. 1). The first peak (peak I) which eluted off the column at around 0.15 mM NaCl, bound [^{3}H]PDBu, but showed partial dependence on PS and Ca^{2+} for activity. The second peak (peak II) appeared entirely independent of the addition of lipids and Ca^{2+}. Characterization of this second peak revealed the absence of c-AMP dependent protein kinase. We tried to use a discontinuous gradient in the DE-52 elution. The elution profile using two major NaCl concentrations i.e. 0.14 M and 0.35 M successively, revealed two kinase activity peaks, with distribution and lipid dependence in agreement with the continuous gradient profile shown in Fig. 1. The total kinase activity, its distribution between peak I and peak II, and the lipid-dependence characteristics, were quite similar when the NaCl concentration, at the first step of the discontinuous gradient, was 0.12, 0.14 or 0.17 M. This demonstates the great difference and the clear division between the two activities. Similar elution profiles were obtained for the membrane extracts (not shown), except that the total activity and the lipid dependence were lower (see below).

Surprisingly, we observed an increase in protein kinase activity when measured at various times after elution. This increase seems to be restricted to peak I (Fig. 2), which was better illustrated over an 8-day period (insert). The increase was considerably high for membranes, the activity was 140 and 420 pmoles/10^7 cells at days 0 and 7 respectively, i.e. a 200% increase. In the same experiment, the activity of peak I of cytosol was 288 pmol of PKC/10^7 cells after elution but reached 426 pmol PKC/10^7 cells 7 days later. This was associated with a complete loss of lipid dependance, and PDBu binding properties. Thus, in the above experiment, activity of peak I from cytosolic and membrane fractions was originally dependent on lipids, 42% and 26% respectively, whereas this lipid dependence considerably decreased at day 2, and no longer existed at day 4.

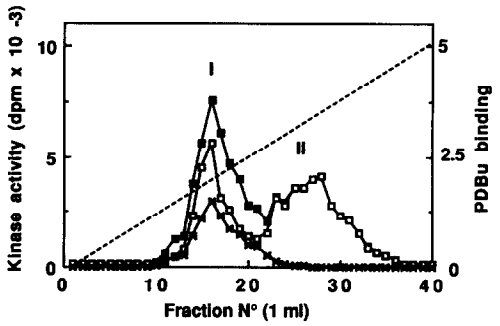

Fig. 1. DEAE-cellulose chromatographic analysis of the cytosolic protein kinase activities. Extract from 1.5 × 10^8 cells was applied to DE-52 columns (1.1 × 5 cm). The column was eluted with 40 ml of a 0–425 mM NaCl linear gradient in buffer B at a flow rate of 1 ml/min. Aliquots of each fraction were tested for protein kinase activity in the precence (■) or the absence of lipids and calcium (□) and PDBu binding (x). The non-specific binding was less than 200 dpm/20 μl fractions. Dotted lines refer to the NaCl concentration. Protein kinase activities are expressed as dpm of ^{32}P incorporated into histone H1/5 min/30 μl aliquot.

It was also intriguing to note that with the dilution of peak I extracts, there was a much higher kinase activity than in the original sample. Thus, a 6-fold dilution with buffer B containing 0.14 M NaCl led to an increase of 110% and 50% of total cytosol and membrane extract activity respectively.

The temporal evolution of protein kinase activity in RPMI 8226 cells and its differential expression as a

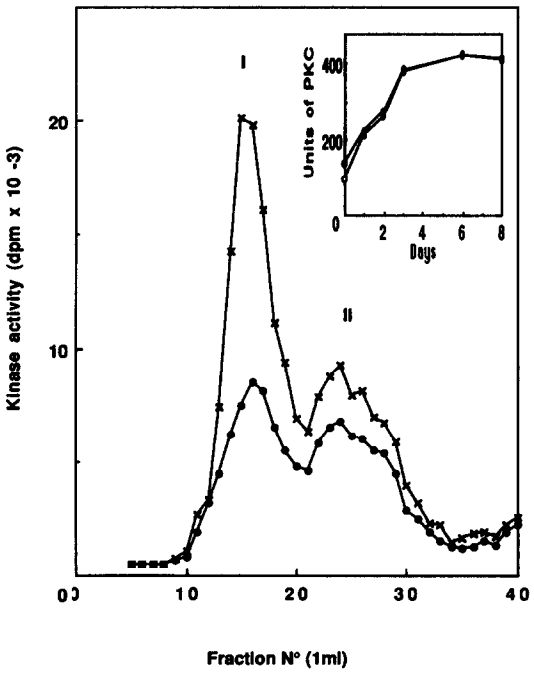

Fig. 2. Temporal evolution of the protein kinase activities. The membrane extracts of 1.5 × 10^8 cells were fractionated on DE-52 columns as in Fig. 1A. Protein kinase activities were assayed immediately (•), or after 48 h (x). Inserts show the total (•) and the lipid-insensitive (○) activity recovered in peak I over 8-day periods, while keeping the various fractions at 4°C.

function of its concentration in the medium made quantification difficult and somewhat imprecise. This led us to choose strictly defined conditions (Table I). Total histone H-1 kinase activity eluting at 0.14 M from cytosol and membrane fraction was 303 ± 28 and 190 ± 20 units/cells, indicating that 38 ± 2% of their total activity was in the membranes. Lipid and Ca^{2+} dependence of the cytosolic and membrane fractions was significantly different at 52 ± 6 and 21 ± 2% respectively. A similar kinase activity was eluted at 0.35 M NaCl for each of the fractions, but the activity seemed to be entirely independent of the addition of lipids and Ca^{2+}. Only peak I could bind (3H) PDBu, with values ranging from 1.85 ± 0.12 ($n=7$) and 0.59 ± 0.05 ($n=6$) pmol/unit of protein kinase activity for cytosolic and membrane fractions respectively.

The above reported activity increase for peak I could be due either to a modification of the enzyme itself or to the extinction of the action of a coeluted-endogenous inhibitor. In this context, we studied the effect of peak I fractions on the expression of a known amount of rat brain PKC. The DE-52 extract from the RPMI 8226 membrane fraction, from fraction 20, produced a significant inhibition of brain PKC. However, this inhibition did not coincide with fractions showing increased kinase activity, as illustrated in Fig. 3. Thus, fraction 15 tripled its activity after 24 h, but did not contain any inhibitor acting on rat brain PKC. In the same way, the DE-52 extract of the RPMI 8226 cytosol eluting beyond fraction 15 produced a significant inhibition of rat brain PKC (maximum inhibition of 50% with fraction 21-24). But, here also, this inhibition did not coincide with fractions showing increased kinase activity (not shown). When tested 4 days after collection, the DE-52 eluate from the cytosolic and membrane fraction of RPMI 8226 showed a similar inhibition profile, except for a slightly lower amplitude, of rat brain PKC (not shown).

Table I

Protein kinase activities in cytosolic and membrane fractions of RPMI 8226 cells

	Cytosol	Membrane	% in membrane
Eluted at 0.14 M NaCl			
+PL	303 ± 28	190 ± 20	38 ± 2%
−PL	172 ± 14	155 ± 20	45 ± 4%
(PL)d	132 ± 26	39 ± 8	24 ± 6%
% (PL)d	52 ± 6%	21 ± 2%	
Eluted at 0.35 M NaCl			
+PL	262 ± 29	183 ± 32	40 ± 3%
−PL	248 ± 30	195 ± 28	43 ± 5%
% (PL)d	4%	0%	

Extract from 1.5 to 2 × 10^8 cells were fractionated on DEAE-cellulose with a discontinuous gradient of NaCl. The protein kinase eluted at 0.14 M and 0.35 M were tested in the presence (+ PL) or absence (− PL) of lipids. (PL)d indicates protein kinase activity that was lipid-dependent. Activities are expressed as nmol/10^{10} cells/min and are means ± SE of 8 experiments with different batches of cells.

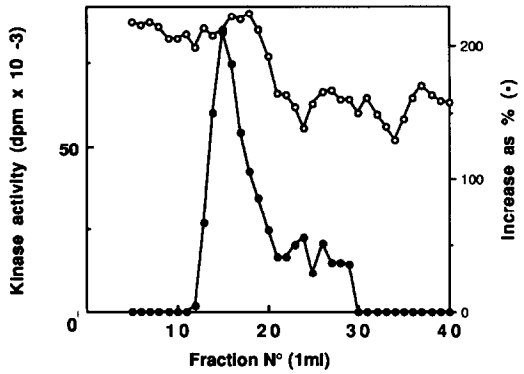

Fig. 3. Effect of the DE-52 eluate of membrane fractions on rat brain protein kinase C. The membrane fraction of 1.5 × 10^8 cells was fractionated on DE-52 columns as in Fig. 1. Aliquots containing 15 μl of each fraction RPMI 8226 cells plus 15 μl of partially purified rat brain PKC (representing 85 000 ± 3000 dpm) were assayed for activity (○). The expressed protein kinase activity is the one observed minus the endogenous activity due to the RPMI 8226 extract (highest endogenous activity was observed in fraction N°13 with 7000 dpm). The lipid independent part of the brain kinase activity was not changed by the addition of RPMI 8226 cell extract, and remained below 7500 dpm. The percentage increase of the endogenous activity of RPMI 8226 extract after 48 h, as observed in Fig. 2, is simultaneously shown (•) and does not correspond to the inhibition of brain PKC.

It was intriguing that the protein kinase activity eluted at 0.14 M NaCl was partly independent of PS and Ca^{2+}. Protein kinase M, a protease-activated form of PKC which is liberated from the lipid and Ca^{2+} to exert its enzymatic activity [12], could be involved in lipid-insensitive kinase activity, and in the loss of this dependence as a function of time. Thus, we looked for such a transformation by rechromatographying the peak eluting at 0.14 M on DE-52 columns, two days after the original elution. In a typical experiment, although lipid dependence had almost completely disappeared, and all activity had greatly increased, the kinase activity was recovered at only 0.14 M. The eventually formed PKM, which is normally eluted at 0.35 M NaCl [12,13] was less than twice the radioactive background, and less than 5% of the total activity. It should be noted that the recovery of total activity (calculated in terms of the whole peak at day + 2), was 90% for the cytosolic fraction, but rather poor (43%) for the membrane fraction. The proteolysis of PKC and transformation into PKM during the test period when Ca^{2+} and PL are present is another possible explanation for partial lipid dependence. In this context, the fractions composing peak I were incubated in conditions exactly similar to those used for PKC determination, with histone, for 10 min at 37°C, before carrying out a new DE-52 chromatography. For both the cytosolic and the membrane fraction, the activity recovered at 0.35 M NaCl was less than 5% of total activity. In fact, all the activity was eluted at a 0.14 M

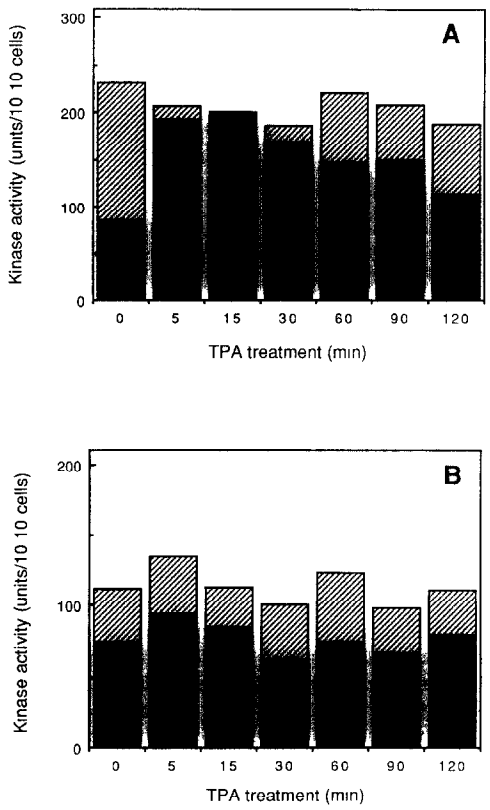

Fig. 4. Effect of TPA on the 0.14 M eluted protein kinase activity. RPMI 8226 cells were incubated in fresh medium at 10^6 cells/ml and then treated with 162 nM TPA for the indicated times before pelleting the cells and separation of the cytosolic (A) and membrane fractions (B). After prepurification on DE-52 columns, protein kinase activity was assayed in the presence (full bar) or the absence (black bar) of lipids and Ca^{2+}. The hatched bar represents the lipid-dependent protein kinase activity.

NaCl concentration. The kinase activity recovery was 67% and 26% for the cytosol and membrane fraction respectively. In this case, the lipid dependence of peak I was not significantly modified by the second ion exchange chromatography.

Finally, we studied the effect of 162 nM TPA on levels and distribution of 0.14 M eluted protein kinase activity (Fig. 4). Surprisingly, TPA treatment does not clearly change total kinase activity levels of the cells. However, it was quite obvious that TPA treatment selectively modified lipid dependence of the cytosolic kinase activity. This lipid dependence was originally 63%, but dropped to 6% and 0% after 5 and 15 min TPA treatment respectively. This was due to an increase in the lipid-independent part of kinase activity. Over 15 min, lipid dependence quickly and partially recovered to 32% after 120 min treatment. On the other hand, the total activity, and the lipid dependence did not seem to be altered in the membrane fraction. The kinase activity eluted at 0.35 M did not show any significant variation in the presence of TPA (not shown).

4. DISCUSSION

Almost all of the kinase activity in normal lymphocytes is recovered in the cytosolic fraction while translocation to the membrane compartment occurs after stimulation, which constitutes an index of in situ activation of the enzyme [1,2,9]. PKC activity from RPMI 8226 cells was eluted from DEAE cellulose at 0.12 M NaCl as can be observed in a large number of other cells. The total amount of PKC activity is similar with B lymphocytes of different species at different stages but there is a clarly marked appearance of PKC in the membrane fraction (Table I).

There was a conspicuous, only partial dependence of lipids for the expression of PKC activity. PKC can be converted, by different types of proteases which are probably of physiological importance, to a form which no longer requires PL for activation [1,2,10]. The action of calpain is quite fulgurant [14]. However, such conventional degradation, either in the eluate of the DE-52 column or in the assay mixture is clearly excluded since rechromatography of the activity after corresponding incubation gave the same elution profile and lipid-dependence as previously observed, without any significant appearance of PKM.

There is a possible presence of endogenous activators which could co-purify, and be responsible for the partial lipid dependence. Thus, after activation of the whole cells by TPA, the recovered enzyme behaved as a membrane constituent, and could not be freed of PL and TPA during the subsequent chromatographic analyses on the DEAE sephacel column [15]. However, it is remarkable that the possible endogenous activator has no effect on rat brain PKC (Fig. 3). (The presence of an extremely tight complex with an activator, without possible exchangeability, can be hypothesized.)

The evolution of the total activity, the loss of PL dependence and the PDBu-binding properties of the 0.14 M eluted activity was surprizing. This suggests the interconversion of a lipid-dependent into a lipid-independent activity, and could reflect conformational changes of the enzyme, or the presence of effectors. However, this temporally increased activity is probably not due to the inactivation of an endogenous conventional inhibitor, otherwise it would have the same effect on rat brain PKC. These facts, and the increased activity after dilution of the samples, illustrate the problems in describing activities, since the proportions in membranes are much higher if ulterior activities are considered.

Activation of the intracellular PKC by phorbol esters lead to translocation of the cytosolic form to the membrane, as observed in many different cell types. This recruitment is usually followed by a protease-mediated transformation of PKC into PKM, which does not contain the hydrophobic domain required for association with the membrane [1,12,15]. The temporal effect of

TPA on RPMI 8226 showed a rapid and complete depletion of cytosolic-dependent activity with no quantitative recovery of the activity in the membrane fraction. More intriguing is the fact that the loss of lipid dependence is not associated with the formation of the 0.35 M migrating PKM, but is associated with an increase of co-migrating lipid-independent protein kinase activity. No explanation can be given at present, but this reveals the atypical behavior of protein kinase C in these cells. One possibility would be that the membrane, saturated with the enzyme, cannot support any additional enzyme.

Alterations in cell growth and differentiation are reflected by differences in PKC subcellular distribution whereas activation of PKC as reflected by translocation of the enzyme from the cytosol to the membrane fraction has been firmly associated to its role in such cellular functions as cell proliferation, viral transformation and leukemia cell differentiation [1-3,16,17]. The subcellular localization (rather than the total level of PKC) seems markedly altered in transformed cells, probably due to translocation, activation, and down regulation [1,16]. More recently, an unusual PKC subcellular distribution, largely membranar, was observed in a fibrosarcoma cell line whose PKC share 4 point mutations, three of them being located in the highly conserved regulatory domain [8]. It is also remarkable that, when the data are available, the lipid-insensitive part of the protein kinase C activity increases in many transformed cells [e.g. 18].

Since neoplastically transformed cells differ from non-transformed cells in parameters related to unrestrained growth, the question could be raised as to whether changes in PKC are generally related to growth rather than specifically to transformation. Our results do not necessarily indicate the direct interaction of unknown activators, nor exclude the possibility that protein kinase has atypical properties. In the former case, a specific lipid-protein interaction would presumably be needed for the activation of PKC.

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REFERENCES

[1] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31-54.
[2] Huang, K.P. (1989) Trends. Neurosci. 12, 425-432.
[3] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1-11.
[4] Bishop, W. and Bell, R. (1988) Oncogene Res. 2, 205-225.
[5] Chiarugi, V., Bruni, P., Pasquali, F., Magnelli, L., Basi, G., Ruggiero, M. and Farnararo, M. (1989) Biochem. Biophys. Res. Commun. 164, 816-823.
[6] Housey, G., Johnson, M., Hsiao, W., O'Brian, C., Murphy, J., Kirschmeier, P. and Weinstein, I. (1988) Cell 52, 343-354.
[7] Persons, D., Wilkison, W., Bell, R. and Finn, O. (1988) Cell 52, 447-458.
[8] Megidish, T. and Mazurek, N. (1989) Nature 342, 807-811.
[9] Vial, H.J., Parant, M.R., Sainte Marie, J., Laurent, A.M. and Le Peuch, C.J. (1989) Leukemia Res. 13, 583-594.
[10] Duperray, C.B., Klein, B., Durie, B., Zhang, X., Jourdan, P., Poncelet, F., Favier, F., Vincent, J., Brochier G. et al. (1989), Blood 73, 566-576.
[11] Parant, M.R. and Vial, H.J. (1990) Anal. Biochem. 184, 283-290.
[12] Murray, A.W., Fournier, A. and Hardy, S.J. (1987) Trends Biochem. Sci. 12, 53-56.
[13] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O. and Sparatore, B. and Horecker (1986) J. Biol. Chem. 261, 4101-4105.
[14] Inomata, M., Imahori, K. and Kawashima, S. (1986) Biochem. Biophys. Res. Commun. 138, 638-649.
[15] Uratsuji, Y., Nakanishi, H., Takeyama, Y., Kishimoto, A. and Nishizuka, Y. (1985) Biochem. Biophys. Res. Commun. 130, 654-661.
[16] Ashendel, C.L., Baudoin, P.A. and Minor, P. (1988) in: Tumor Promoters (Lagenbach, R. et al. eds) Raven, New York, pp. 331-342.
[17] Uratsuji, Y. and Dilorleto, P.E. (1988) J. Cell. Physiol. 136, 431-438.
[18] Wolfson, M., Aboud, M., Ofir, R., Weinstein, Y. and Segal, S. (1986) Int. J. Cancer 37, 589-593.