Multiple mechanisms of protein kinase C inhibition by triphenylacrylonitrile antiestrogens

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The activation of type I (γ), II (β) and III (α) protein kinase C (PKC) subspecies by phosphatidylserine (PS) and diacylglycerol (DAG) is inhibited by micromolar concentrations of triphenylacrylonitrile (TPE) antiestrogens. TPE *A* (with *p*-hydroxy and *p*-diethylaminoethoxy groups on the 3- and 3′-phenyl rings, respectively) interacts with PS-vesicles as well as with the regulatory domain of PKC, probably at a site different from the Ca^{2+} and DAG binding sites. The interaction of TPE *A* with the regulatory domain of enzyme is very slow. Apparently, TPE *A* does not interact with the catalytic domain of PKC. In contrast, another TPE derivative, TPE *B* (with a *p*-hydroxy group on each of the three phenyl rings) inhibits the enzyme activity in a competitive manner with respect to ATP, suggesting that this TPE interacts with the catalytically active site of the enzyme. It seems likely that various TPE antiestrogen derivatives may exert their inhibitory action on PKC by multiple different mechanisms.

Protein kinase C; Antiestrogen; Triphenylethylene; Human breast cancer

1. INTRODUCTION

Triphenylethylenes (TPE derivatives), a well-known chemical family (for a review, see [1]), bind to estrogen receptor (ER) and exhibit either estrogenic, antiestrogenic and/or antitumoral activities [1–3]. In spite of the widespread use of TPE tamoxifen for treatment of hormone-dependent breast cancer, its mechanism of action remains to be elucidated. Pharmacological studies of several members of a related chemical family, triphenylacrylonitriles, have shown that their ability to inhibit human breast cancer cell proliferation depends largely on the structure and position of substituents on the triphenyl skeleton [4–7]. Since it is generally accepted that interaction of TPEs with ER is not the sole basis of their antiproliferative effect on human breast cancer cells [7–10], additional mechanisms appear to be involved in the observed antitumoral action (for a review, see [11]).

It has been proposed that an important target of TPEs is protein kinase C (PKC) [12] which plays a critical role in growth regulation (for reviews, see [13,14]). To understand better the mechanism of inhibition of PKC by TPEs, we have examined the kinetics of the inhibitory actions of two triphenylacrylonitrile derivatives which represent different chemical subclasses (Fig. 1) (*A* and *B* correspond to TPEs *1* and *3* in a previous report [15].) TPE *A* (with one phenolic and one basic residue) profoundly inhibits, in the nanomolar concentration range, estrogen-induced proliferation of MCF-7 cells, an ER-positive cell line derived from human breast cancer metastasis, and this inhibition is overcome by estrogen [7]. TPE *B* (with three phenolic residues) exerts only partial inhibition on this cell line [6,7]. Both TPEs at the micromolar concentration range, inhibit ER-positive as well as ER-negative breast cancer cell lines, and this inhibition is not overcome by estrogen (estrogen irreversible effect) [7].

Our earlier study [15] has suggested that TPEs inhibit PKC by more than a single mechanism. Further evidence for interaction of these TPEs with PS-vesicles and with the regulatory and catalytic domains of PKC will be presented herein.

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Abbreviations: TPE, triphenylethylene derivative; ER, estrogen receptor; PKC, protein kinase C; PKM, a catalytically active fragment of PKC; PS, phosphatidylserine; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; DO, diolein

2. MATERIALS AND METHODS

2.1. *Chemicals and materials*

The preparation of TPEs and their analytical characteristics have been described previously [5,7]. The isomeric purity was checked before use by HPLC analysis as described [7].

2.2. *Enzyme purification*

PKC was purified from the rat brain soluble fraction and separated into three types, type I, II and III, as described [16].

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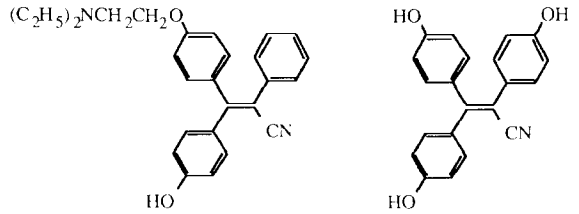

TPE A TPE B

Fig. 1. Structures of TPE derivatives.

2.3. *Assay of PKC*

Unless otherwise indicated, the standard reaction mixture (0.125 ml) contained buffer A (20 mM Tris-HCl at pH 7.5), 10 μM [γ-^{32}P]ATP (400–500 cpm/pmol), 5 mM magnesium acetate, 0.01 mM EGTA and 0.01 mM EDTA (these EGTA and EDTA were from the enzyme fraction), 200 μg/ml calf thymus H1 histone, 0.1 mM $CaCl_2$, 8 μg/ml phosphatidylserine (PS) vesicles, 0.8 μg/ml DO, PKC and, where indicated, TPE. TPEs were dissolved in DMSO. PS and diolein (DO) were stored in chloroform solution. The PS solution was evaporated under a stream of nitrogen (when DO was used, it was mixed first with PS in chloroform). The residue was resuspended in ice-cold buffer A, vigorously mixed using a vortex mixer for 1 min, and sonicated with a tip sonicator for 1 min at 0°C. TPE solutions in DMSO were diluted in buffer A and, when indicated, vigorously mixed (vortex) for 1.5 min with an aliquot of the sonicated PS-vesicles (the final DMSO concentration in the incubation was 5% (v/v)). The PS-TPE solution was added to the reaction mixture, and the phosphorylation reaction was started by the addition of PKC (approx. 0.05 μg). The incubation was carried out for 3 min at 30°C under gentle shaking (40 cycles per min). The radioactivity of acid-precipitable materials was determined by liquid scintillation. All experiments were carried out at least twice.

2.4. *Assay of Ca^{2+}- and PS-independent activity of PKC*

With protamine sulfate as phosphate acceptor, PKC exhibits full enzymatic activity in the absence of PS, DAG and Ca^{2+} [17]. This protein kinase activity was determined with 400 μg/ml protamine sulfate in the presence of EGTA (0.5 mM in final concentration) instead of Ca^{2+}, PS and DO. TPE was added directly to the reaction mixture (the final DMSO concentration in the incubation mixture was 5% (v/v)).

2.5. *Preincubation experiments of TPEs with PKC*

The preincubation mixture in ice-cold buffer A (0.105 ml) contained 0.01 mM EGTA, 0.01 mM EDTA, 0.7% glycerol (v/v), 29.8 μg/ml bovine serum albumin (that stabilizes PKC without affect on TPE action), TPE or DMSO (5.9% (v/v)) with or without 0.5 μg/ml PS. TPE was added directly (TPE and PS-vesicles were not mixed vigorously) to this mixture (the final DMSO concentration in the preincubation mixture was 5.9% (v/v)). The preincubation was started by the addition of PKC (approx. 0.3 μg) and carried out at 0°C for various periods of time. The phosphorylation reaction was then initiated by the addition of other cofactors and substrate, and the incubation was carried out for 2 min at 30°C as described above (the final volume was 0.125 ml).

3. RESULTS

3.1. *Inhibition of PKC by TPEs*

In the presence of Ca^{2+} and PS (plus or minus DAG), type I, II and III PKC subspecies were all inhibited by TPEs *A* and *B* in a concentration-dependent manner. TPE *A* inhibited PKC with an IC_{50} of 1–5 μM, whereas TPE *B* inhibited the enzyme at 20-fold higher concentrations. Kinetic analysis indicated that the TPEs interact with PS-vesicles and/or PKC but not with Ca^{2+} and DAG (see below). This inhibition was not competitive with respect to Ca^{2+} nor to DAG. Since essentially similar results were obtained for type I, II and III PKC, only the results obtained for type III PKC, which is commonly present in mammalian tissues and cell types including human breast cancer cells (PKC III (α) is one of the PKC subspecies expressed in human breast cancer cells (Eric Bignon et al., manuscript in preparation)), will be given below.

3.2. *Interaction with phospholipid*

PS-vesicles and TPE solutions, prepared separately as specified in section 2, were vigorously mixed using a vortex mixer for various periods of time. This solution was then added to the reaction mixture containing Ca^{2+}, Mg^{2+}, ATP and H1 histone in buffer A. The phosphorylation reaction was started by the addition of PKC and carried out for 3 min. It was found that TPE *A* markedly inhibited the reaction only after being vigorously mixed with PS-vesicles for a certain period of time (Fig. 2). It is unlikely that this profound inhibitory action of TPE *A* was simply due to its direct interaction with the PKC molecule or with other components in the reaction mixture, because TPE *A* showed practically no effect unless it was vigorously mixed with PS-vesicles. The presence of higher concentrations of PS during its mixing with TPE *A* fully overcame this inhibitory action according to a sigmoidal PS dose response curve, which may reflect PS-TPE interaction (Fig. 3). Other procedures such as sonication of the

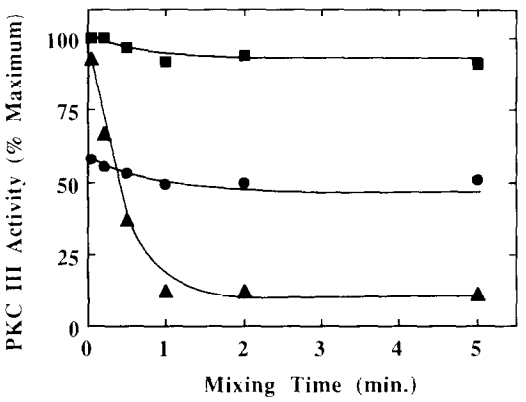

Fig. 2. PKC III activity as a function of mixing time (vortex) of PS-vesicles with TPEs. PS-vesicles and TPE solutions were prepared separately as specified in section 2, and mixed vigorously using a vortex mixer for various periods of time. Then, this solution was added to the reaction mixture containing Ca^{2+}, Mg^{2+}, ATP, H1 histone in buffer A. When TPE was mixed with vehicles, PS-vesicles were added during the phosphorylation reactions. The phosphorylation reaction was started by the addition of PKC, and carried out for 3 min. (▲) 60 μM TPE *A*, (●) 100 μM TPE *B*, (■) vehicle alone. The results are normalized to the maximum activity that was obtained in the presence of Ca^{2+} and lipids.

TPE-PS solution, which facilitate the interaction of TPE with phospholipid, also resulted in profound inhibition of the reaction (data not shown). The results suggest that TPE *A* interacts with the phospholipid-vesicles and thereby inhibits PKC activation.

In contrast, the inhibitory action of TPE *B* did not depend on its premixing with PS-vesicles or on the PS concentration (Figs 2 and 3). A possible mechanism of this inhibition is described below.

3.3. *Interaction with regulatory domain of PKC*

In the next set of experiments, TPE and PKC were preincubated first (in the absence of PS) at 0°C for various periods of time. PKC activity was then determined in a 2 min incubation assay. The reaction was started by the addition of this enzyme-TPE solution to the incubation mixture containing Ca^{2+}, Mg^{2+}, PS, ATP and H1 histone as a phosphate acceptor in buffer A. As shown in Fig. 4A, without preincubation (time 0), TPE *A* showed no effect on enzyme activity, suggesting that this compound does not significantly interact with PS-vesicles nor with other components during the 2 min PKC assay. When the preincubation was prolonged, however, the enzyme activity decreased, and after 40 min preincubation, the enzyme showed practically no activity. Although the kinetics of this inhibition was very slow, it is unlikely that TPE *A* denatured PKC irreversibly, because, when the substrate, H1 histone, was replaced by protamine sulfate, which does not require Ca^{2+}, PS and DAG for enzymatic reaction [17], the enzyme was almost fully active under the prolonged preincubation (Fig. 4B).

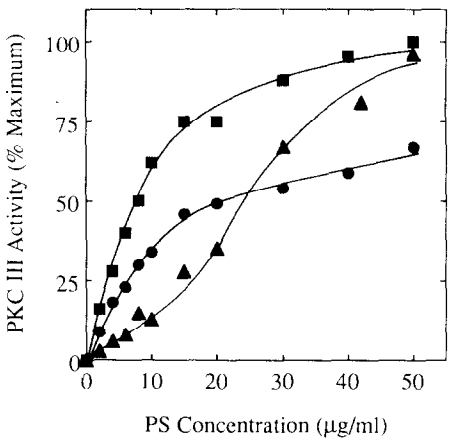

Fig. 3. PKC III activity as a function of PS concentration in the presence of TPEs. PS-vesicles and TPE solutions were prepared separately as specified in section 2, and mixed vigorously using a vortex mixer for 1.5 min. Then, this solution was added to the reaction mixture containing Ca^{2+}, Mg^{2+}, ATP, H1 histone in buffer A. When TPE was mixed with vehicles, PS-vesicles were added during the phosphorylation reactions. The phosphorylation reaction was started by the addition of PKC, and carried out for 3 min. (▲) 60 μM TPE *A*, (●) 100 μM TPE *B*, (■) vehicle alone. The results are normalized to the maximum activity that was obtained in the presence of Ca^{2+} and lipids.

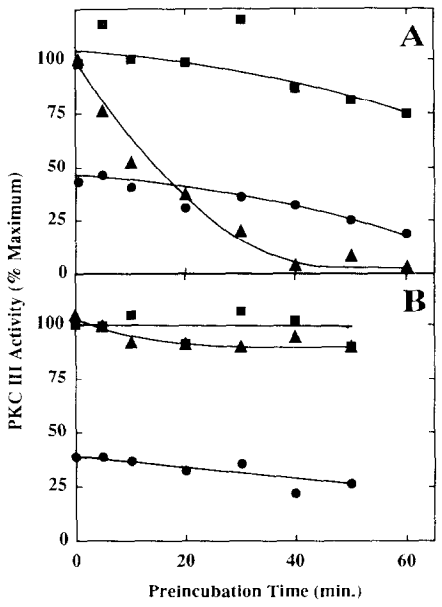

Fig. 4. PKC III activity as a function of preincubation time of TPEs with enzyme. TPEs and PKC were preincubated (in the absence of PS vesicles) at 0°C for various periods of time under gentle shaking. The phosphorylation reaction was started by the addition to this solution of the reaction mixture containing (A) Ca^{2+}, Mg^{2+}, PS, ATP and H1 histone in buffer A, or (B) ATP, Mg^{2+} and protamine sulfate in buffer A, under the conditions specified in section 2. The reaction was carried out for 2 min. (▲) 60 μM TPE *A*, (●) 100 μM TPE *B*, (■) vehicle alone. The results are normalized to the activity that was obtained in the presence of Ca^{2+} and lipids (A), or 0.5 mM EGTA (B).

Similarly, TPE *A* did not significantly inhibit PKM, a catalytically active fragment of PKC. The results indicate that TPE *A* may also interact slowly with the regulatory domain of PKC, thereby preventing subsequent activation of the enzyme by Ca^{2+} and lipid cofactors. After 40 min preincubation, the activity of the

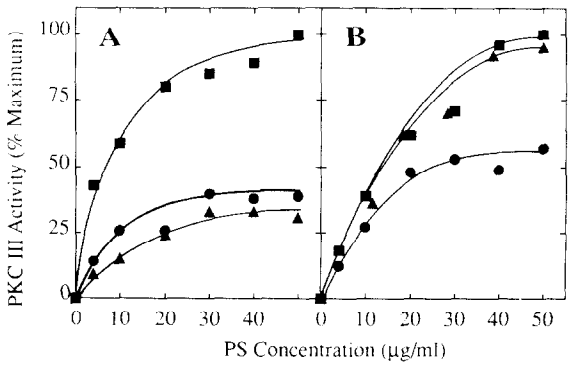

Fig. 5. Effect of PS concentration on inhibition of PKC activation by TPEs. (A) Various amounts of PS-vesicles were added after 40 min preincubation of PKC with TPEs; (B) various amounts of PS-vesicles were added during 40 min preincubation of PKC with TPEs (TPEs and PS vesicles were not vigorously premixed). Other conditions were identical to those given in Fig. 3. (▲) 60 μM TPE *A*, (●) 100 μM TPE *B*, (■) vehicle alone. The results are normalized to the maximum activity that was obtained in the presence of Ca^{2+} and lipids.

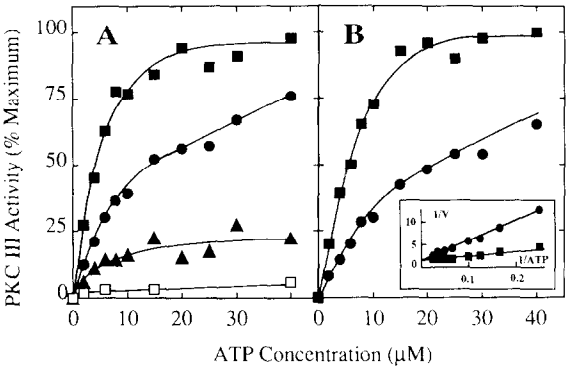

Fig. 6. PKC activity as a function of ATP concentration. PKC activity was determined as described in section 2 in the presence of (A) Ca^{2+}, Mg^{2+}, PS, and H1 histone in buffer A, or (B) EGTA, Mg^{2+}, and protamine sulfate in buffer A (double-reciprocal plot (Inset), 1/PKC activity ($1/V$: $nmol^{-1} \cdot min$) as a function of 1/ATP concentration (μM^{-1})). (■) Control, (□) 0.5 mM EGTA, (▲) 60 μM TPE *A*, (●) 100 μM TPE *B*. Similar results were obtained with types I and II PKC. The K_m values of ATP for type I, II and III PKC were 6.75 ± 0.04, 6.74 ± 0.59 and 6.67 ± 0.04 μM with H1 histone, and 10 ± 3, 18 ± 1 and 11 ± 3 μM with protamine sulfate as a substrate, respectively. The results are normalized to the maximum activity that was obtained in the presence of Ca^{2+} and lipids (A) or 0.5 mM EGTA (B).

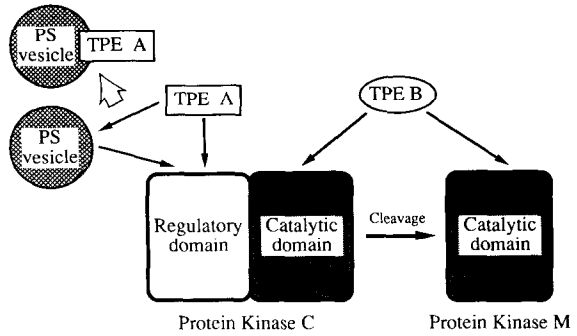

Fig. 7. Schematic representation of TPE interaction with PKC, PKM and PS-vesicles. The solid arrows represent possible main interactions between these components. The interaction of TPE *A* with PKC may induce a conformational change of the enzyme, but its binding site to the PKC molecule remains undefined. TPE *B* probably interacts with the ATP-binding site of PKC and PKM.

enzyme could not be restored by the subsequent addition of PS-vesicles (Fig. 5A). However, the presence of PS during the preincubation prevented the enzyme inactivation by TPE *A* (Fig. 5B).

Unlike TPE *A*, TPE *B* inhibited phosphorylation of both H1 histone (Fig. 4A) and protamine sulfate (Fig. 4B) with or without preincubation with the enzyme. This inhibition was not significantly affected by PS-vesicles under various conditions tested (Fig. 5).

3.4. *Interaction with catalytic domain of PKC*

The phosphorylation of protamine sulfate, which does not require Ca^{2+}, PS and DAG, was apparently insensitive to TPE *A*, but significantly inhibited by TPE *B* (Fig. 4B). Similar results were obtained with a catalytically active fragment of PKC (PKM), suggesting that TPE *B*, but not TPE *A*, interacts directly with the catalytic domain of enzyme. Inhibition of PKC by TPE *B* was competitive with ATP with either H1 histone or protamine sulfate being used as a phosphate acceptor (Fig. 6A and B), whereas inhibition by TPE *A* was not reversed by increasing ATP concentrations. The catalytic subunit of cyclic AMP-dependent protein kinase was insensitive to these TPEs under comparable conditions.

4. DISCUSSION

The present studies have shown that TPE *A* interacts with PS vesicles as well as with the regulatory domain of PKC, thereby inhibiting the enzyme activation as schematically shown in Fig. 7. This interaction of TPE *A* with PS vesicles and PKC appears to proceed slowly. Although increasing the concentration of PS vesicles apparently overcomes the inhibitory component of TPE *A* in its interaction with PS vesicles, there is no indication that TPE *A* competes with PS vesicles for the same site of the regulatory domain of enzyme. It is possible that TPE *A* inhibits the association of enzyme with PS vesicles by altering the phospholipid lamellar structure, although the precise mechanism of PKC activation by phospholipid still remains largely unexplored. On the other hand, the inhibitory component of TPE *A* in its interaction with PKC is an apparently irreversible process, which involves some conformational change of the enzyme molecule. The binding of TPE *A* to PKC appears to be prevented by prior association of the enzyme with PS vesicles. A conformational change of the PKC molecule by membrane phospholipid has been suggested by Bazzi and Nelsestuen [18] and Newton and Koshland, Jr. [19].

TPE *B*, like a certain diphenylethylene analogue [20], interacts with the catalytic domain of PKC. TPE *A* at higher concentrations (in the range of 100–600 μM) may also interact with the catalytic domain of enzyme, and inhibits its PS- and Ca^{2+}-independent activity [21].

The inhibitory action of TPEs *A* and *B* on PKC described above apparently parallels their observed ER-independent action on human breast cancer cell proliferation [7]. Although the plasma tamoxifen level varies greatly depending on the patient and therapy conditions, the tissue concentration of this compound during the cancer treatment has been estimated to be sufficiently high to inhibit PKC [22,23]. The antitumor action of TPEs may, therefore, probably result from several mechanisms, involving their interaction with ER and also with other molecular targets, such as PKC and Ca^{2+}/calmodulin-dependent protein kinase [3] which may also play a role in growth regulation.

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REFERENCES

[1] Miquel, J.-F. and Gilbert, J. (1988) J. Steroid Biochem. 31, 525–544.
[2] Jordan, V.C. (1984) Pharmacol. Rev. 36, 245–276.
[3] Sutherland, R.L., Watts, C.K.W., Hall, R.E. and Ruenitz, P.C. (1987) J. Steroid Biochem. 27, 891–897.
[4] Pons, M., Michel, F., Bignon, E., Crastes de Paulet, A., Gilbert, J., Miquel, J.-F., Précigoux, G., Hospital, M., Ojasoo, T. and Raynaud, J.-P. (1984) in: Progress in Cancer Research and Therapy (Bresciani et al. eds) vol. 31, pp. 27–36, Raven, New York.
[5] Pons, M., Michel, F., Crastes de Paulet, A., Gilbert, J., Miquel, J.-F., Précigoux, G., Hospital, M., Ojasoo, T. and Raynaud, J.-P. (1984) J. Steroid Biochem. 20, 137–145.
[6] Bignon, E., Pons, M., Gilbert, J. and Crastes de Paulet, A. (1988) J. Steroid Biochem. 31, 877–885.
[7] Bignon, E., Pons, M., Crastes de Paulet, A., Doré, J.-C., Gilbert, J., Abecassis, J., Miquel, J.-F., Ojasoo, T. and Raynaud, J.-P. (1989) J. Med. Chem. 32, 2092–2103.
[8] Bardon, S., Vignon, F., Derocq, D. and Rochefort, H. (1984) Mol. Cell. Endocrinol. 35, 89–96.
[9] Taylord, C.M., Blanchard, B. and Zava, D.T. (1984) Cancer Res. 44, 1409–1414.
[10] Katzenellenbogen, B.S., Norman, M.J., Eckert, R.L., Pelt, S.W. and Mangel, W.F. (1984) Cancer Res. 44, 112–119.
[11] Borgna, J.-L. (1987) in: Breast-Diseases Senologia, vol. 2, pp. 127–136, Masson, Paris.
[12] O'Brian, C.A., Liskamp, R.M., Solomon, D.H. and Weinstein, I.B. (1985) Cancer Res. 45, 2462–2465.
[13] Nishizuka, Y. (1984) Nature 308, 693–697.
[14] Nishizuka, Y. (1988) Nature 334, 661–665.
[15] Bignon, E., Ogita, K., Kishimoto, A., Gilbert, J., Abecassis, J., Miquel, J.-F. and Nishizuka, Y. (1989) Biochem. Biophys. Res. Commun. 163, 1377–1389.
[16] Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) J. Biochem. (Tokyo) 103, 759–765.
[17] Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[18] Bazzy, M.D. and Nelsestuen, G.L. (1988) Biochemistry 27, 7589–7593.
[19] Newton, A.C. and Koshland, Jr., D.E. (1989) J. Biol. Chem. 264, 14909–14915.
[20] Bignon, E., Kishimoto, A., Pons, M., Crastes de Paulet, A., Gilbert, J., Miquel, J.-F. and Nishizuka, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1471–1478.
[21] O'Brian, C.A., Ward, N.E. and Anderson, B.W. (1988) J. Natl. Cancer Inst. 80, 1628–1633.
[22] Murphy, C., Fotsis, T., Pantzar, P., Adlercreutz, H. and Martin, F. (1987) J. Steroid Biochem. 26, 547–555.
[23] Jordan, V.C., Bain, R.R., Brown, R.R., Gosden, B. and Santos, M.A. (1983) Cancer Res. 43, 1446–1450.