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Identification of a novel protein kinase C inhibitor in microsomes from phytohaemagglutinin activated human peripheral blood mononuclear cells

Enrico Gandini, Patrizia Orlando, Rita Selvatici, Alessandra Balboni, Sara Boninsegna and Michele Rubini

Institute of Medical Genetics, University of Ferrara, Ferrara, Italy

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A peptide inhibiting either corpuscolate or purified PKC has been identified from microsomes of PHA-activated human PBMC but it is not detectable in microsomes of resting PBMC. The peptide was obtained from a microsomal preparation in an oligomeric form that could be transformed into a monomeric form by β-MSH. The active peptide (IN) was retained on a PC-11 chromatographic column and could be eluted with NaCl. IN is ineffective on PKC-dependent protamine phosphorylation of protamine and on Ca^{2+} and phospholipid-independent activity generated by mild hydrolysis with trypsin of PKC. Ca^{2+} binding is permissive for IN activity IN inhibits particulate PKC in PHA-activated PBMC, but is ineffective after TPA activation. All these data indicate that IN acts at the regulatory domain of PKC.

PKC; Inhibitor; Regulatory domain

1. INTRODUCTION

Protein kinase C belongs to the class of second messenger-dependent protein kinases and plays a key role in extracellular signal transduction, cell proliferation and cell differentiation [1,2]. At least 10 molecular subspecies of PKC have been described, with differential tissue expression, specific intracellular localization and distinct enzymological characteristics [3]. PKC is made up of a regulatory and a catalytic domain; the first contains the Ca^{2+}-dependent, phospholipid- and DAG-binding site, and the second, the ATP-binding site. One of the most outstanding characteristics of PKC is that it is physiologically active only when located in the plasma membrane, i.e. when it is associated with the proper lipid component [1]. Anionic phospholipids (phosphatidylserine and phosphatidylinositol) present in cellular membranes are required for PKC activation. The enzyme–phospholipid interaction is Ca^{2+}-dependent and a ternary non-active enzyme + Ca^{2+} + phospholipid complex is formed and translocated to the membrane. The binding of DAG (or phorbol esters) eventually leads through conformational changes to the enzyme's activation [4]. The binding to lipids is critical [4]. Epand suggests as a general rule that positively charged substances inhibit PKC activation, while negatively charged substances activate it [1].

In the present paper we report that the supernatant of a sonicated microsomal fraction from PHA-activated human PBMC contains a peptide that inhibits PKC 'in vitro', by interacting with the regulatory domain of the enzyme.

In 'in vivo tests', the peptide inhibits particulate PKC in PHA-activated PBMC, but is ineffective in TPA-activated cell cultures.

Correspondence address E. Gandini, Institute of Medical Genetics, via L. Borsari 46, 44100 Ferrara, Italy. Fax: (39) (532) 47618.

Abbreviations: PKC, protein kinase C; PKA, protein kinase A; PHA, phytohaemagglutinin; β-MSH, β-mercaptoethanol; PL, phospholipid; PBMC, peripheral blood mononuclear cells; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; DAG, diacylglycerol, PS, phosphatidylserine; SptMF, supernatant of sonicated microsomal fraction.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

PBMC were isolated from healthy donors by Ficoll/Paque (Pharmacia) density gradient centrifugation [5]. PBMC were suspended at 1×10^6 cells/ml in Iscove Dulbecco modified medium (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco), 100 U/ml penicillin and 100 U/ml gentamycin and were incubated at 37°C in 5% CO_2 humidified atmosphere. TPA (Sigma) and PHA (Burroughs Wellcome) were administered at 50 ng/ml and 1 μg/ml, respectively.

2.2. *Protein kinase C activity*

Cytosolic and particulate PKC were partially purified by DEAE-cellulose (Whatman) chromatography as already reported [6–8]. PKC activity was assayed by a modification of the procedure of Kishimoto et al. [9] according to Mellon et al. [10], using histone III S (Sigma) as a substrate. One unit of PKC activity was defined as the amount that causes the incorporation of 1 nmol of [^{32}P]ATP (300 Ci/mmol) (Amersham) in the substrate under the experimental conditions. Protamine (Sigma) was also tested as a substrate at a 0.01, 0.1 and 1 μg

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concentration. Protein kinase A activity was measured according to Sullivan et al. [23] using casein II (Sigma) as a substrate.

2.3. *Preparation of SptMF*

PBMC were suspended in 0.25 M sucrose, 1 mM EDTA and 1 mM leupeptin at a density of 100×10^6 cells/ml and disrupted in a glass–Teflon homogenizer. After a series of 50 strokes the percentage of intact cells was monitored under the microscope. The procedure was stopped when intact cells were less than 5%. The subcellular fractions were separated by initial centrifugation at $600 \times g$ for 10 min. Supernatant I was collected and centrifuged at $12{,}000 \times g$ for 10 min, supernatant II centrifuged at $100{,}000 \times g$ for 1 h. The pellet (microsomal fraction) was resuspended in 1 ml of 0.25 M sucrose and 1 mM EDTA, sonicated (one pulse for 10 s) and centrifuged again at $100{,}000 \times g$ for 1 h. The supernatant III, SptMF, that appeared to be perfectly clear, was boiled for 45 min. Protein determination was performed according to Bradford [11] using bovine serum albumin as a standard. Aliquots corresponding to about 75–80 ng of protein were added to 50×10^6 PHA activated PBMC and particulate PKC was tested after 30 min.

2.4. *Ion-exchange chromatography*

500 μl of SptMF containing approximately 150 μg protein, were loaded onto a P11-cellulose phosphate (Whatman) column (0.5×2.5 cm) (previously equilibrated with 5 ml of 0.5 M sodium acetate pH 6 and 10 ml of 0.05 M sodium acetate pH 6) and adsorbed proteins were eluted with a 20 ml linear 1–2 M NaCl gradient. The fractions (1 ml each) were concentrated by Amicon Microconcentrators-3 to approximately 500 μl and collected in 0.05 M sodium acetate. 100 μl of each fraction were tested on one unit of cytosolic semipurified PKC. The fractions carrying inhibitory activity contained from 8 to 12 ng of protein. In the particulate PKC assay, the remaining 400 μl of inhibiting fraction (40–50 ng of protein) were added to 50×10^6 PHA-activated PBMC.

2.5. *Trypsin digestion*

The inhibiting activity of eight different IN preparations from the same donor was tested on semipurified PKC before and after trypsin treatment (1 mg/ml, 2 h at 37°C). Trypsin (1 mg/ml) + soybean (20 μg/ml) (Gibco) were added to semipurified cytosolic PKC as control of trypsin-treated IN samples.

2.6. *Mild-hydrolysis of protein kinase C with trypsin*

DEAE-cellulose semipurified PKC was partially digested with 1 mg/ml of trypsin (Sigma) for 10 min at 37°C to cleave the regulatory domain. The digestion was stopped by the addition of bovine serum albumin 10 mg/ml. The digested PKC was stored at 4°C and used within one hour.

2.7. *SDS-polyacrylamide gel electrophoresis*

Electrophoretic analysis of the active fraction was performed in SDS-polyacrylamide gel electrophoresis (15% acrylamide) under standard conditions [12] and stained by the Silver assay [12]. In some experiments β-MSH was removed from the electrophoretic sample buffer.

3. RESULTS

3.1. *Isolation of PKC inhibitor from PHA-activated PBMC microsomes*

After sonication of the microsomal preparation and centrifugation at $100{,}000 \times g$, the obtained supernatant, SptMF, contained a factor capable of inhibiting the activity of particulate PKC translocated to the membrane of PHA-activated PBMC. In several experiments the mean particulate PKC activity in PHA-activated PBMC treated with SptMF was significantly lower than PKC values obtained in PHA-activated cultures to which SptMF was not added (Table I). The degree of inhibition ranged from 18% to 100%. SptMF which had not been previously boiled was inactive on particulate PKC. The inhibitory activity of SptMF was completely retained by a PC-11 chromatographic column and recovered by elution with a linear 1–2 M NaCl gradient. Peak activity was detected between 1.35–1.50 M NaCl.

Table I

Inhibitory effect of heated SptMF on particulate PKC

Treatment	Particulate PKC (U)
PHA-activated PBMC	1.56 ± 0.25
Same, plus heated SptMF	0.38 ± 0.11

In 13 experiments particulate PKC values from PHA-activated PBMC cultures (50×10^6 cells) were compared to PHA-activated PBMC cultures to which 75 ng SptMF had been added. Mean and S.E. are presented ($P < 0.001$).

3.2. *Molecular properties of the inhibitory factor (IN)*

Tryptic digestion of active fractions on semipurified cytosolic PKC removes the inhibitory activity, suggesting that the inhibitory factor (IN) is a protein (Table II). From an average 100 million cells 8–12 ng of IN were obtained.

Ultrafiltration with 3,000 M.W. cut-off Amicon filters, completely retained the activity, while it passed through filters with a 10,000 M.W. cut-off, indicating that the active protein M.W. was between 3,000 and 10,000 M.W. SDS-gel electrophoresis of the fractions eluted from the PC11 column, exhibiting the highest PKC inhibitory activity showed a single band of approximately 1,500 Da (Fig. 1a). This result contradicts the experiments indicating that the inhibitory activity was retained by a filter with a 3,000 M.W. cut-off. When β-MSH was removed from the electrophoretic sample buffer a fuzzy, single band was observed at about 6,500 M.W. (Fig. 1b). These results indicate that in its native form IN is an oligomeric protein and that β-MSH cleaves the disulfide bridges that keep the subunits together. β-MSH is also present in the DEAE elution

Table II

Effect of trypsin digestion on the inhibitory effect of IN on DEAE-cellulose semipurified PKC

Treatment	PKC (U)	
	Native	After trypsin digestion
Control	1.45	1.58
IN	0.78 ± 0.02 (inhibition = 46%)	1.30 ± 0.03 (inhibition = 17%)

PKC was assayed prior ('native') and after trypsin treatment. IN: active fraction eluted from P11-cellulose phosphate column. The data exposed are the mean values ± S.E. of 8 experiments. For details see the experimental section.

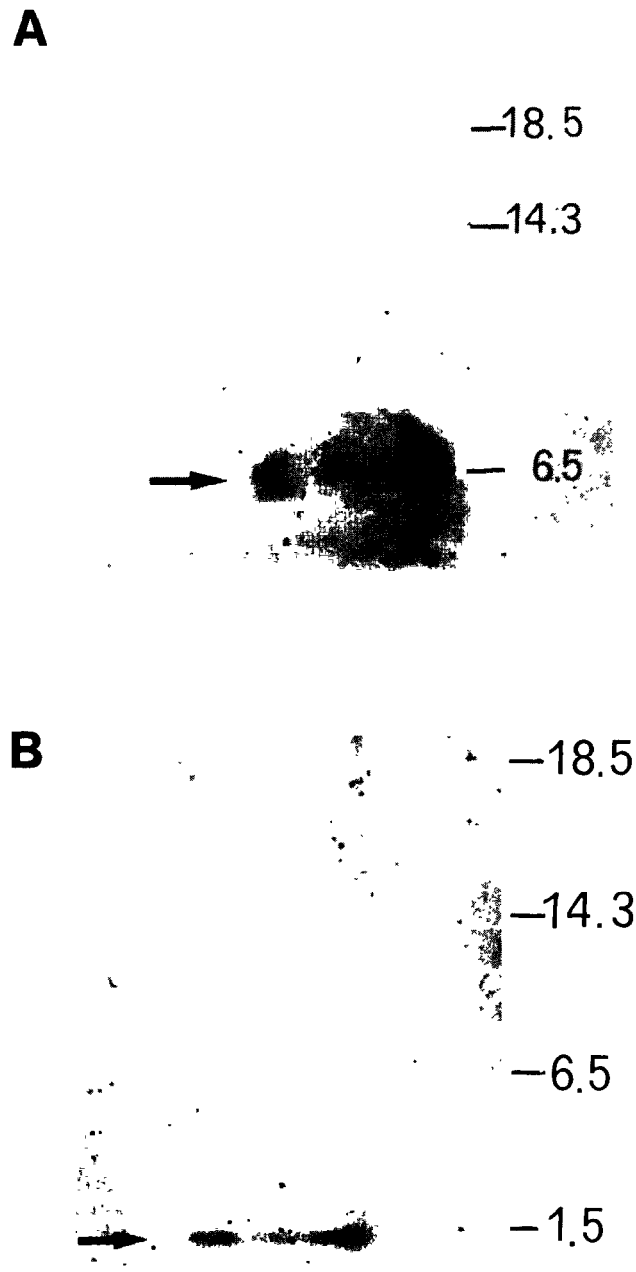

Fig. 1. Silver-staining of SDS-polyacrylamide gel electrophoresis of IN. (A) Under standard conditions; (B) without β-MSH. The active fraction eluted from the P11-cellulose phosphate column was employed.

buffer of semipurified PKC and therefore might be responsible for the transformation of the native IN (6,500 M.W.) to the 1,500 M.W. The inhibitory activity of IN 'in vitro' was studied in a dose-dependent experiment. A linear increase of inhibition from 0 to 85% of PKC activity was observed, up to 24 ng of IN. Higher doses up to 40 ng did not essentially increase the inhibition (Fig. 2).

3.3. *Inhibitory properties of IN*

IN does not significantly affect cyclic-AMP-dependent kinase (PKA) (Fig. 2). Inhibition of DEAE-cellulose semipurified PKC was investigated following two main approaches. (1) The use of protamin. This substrate does not require Ca^{2+} or PL and is phosphorylated by PKC without the activation of the regulatory domain [13]. In this case IN was ineffective (Table IIIA). (2) Mild hydrolysis of PKC with trypsin that cleaves the regulatory domain and generates Ca^{2+}- and PL-independent enzymatic activity. After mild trypsinization of PKC, IN was ineffective on the Ca^{2+}- and PL-independent activity. The tryptic digestion was interrupted when Ca^{2+}- and PL-dependent PKC activity was equivalent to the Ca^{2+}- and PL-independent activity. TPA may substitute for DG and the IN activity was not modified (data not shown). When PS and DG were removed from the reaction mixture the percentage of PKC inhibition by IN was unmodified. When Ca^{2+} was omitted from the reaction no significant PKC inhibition was observed, suggesting that at least 5 mM Ca^{2+} was required for IN activity (Fig. 3). A gradual increase of the sub-

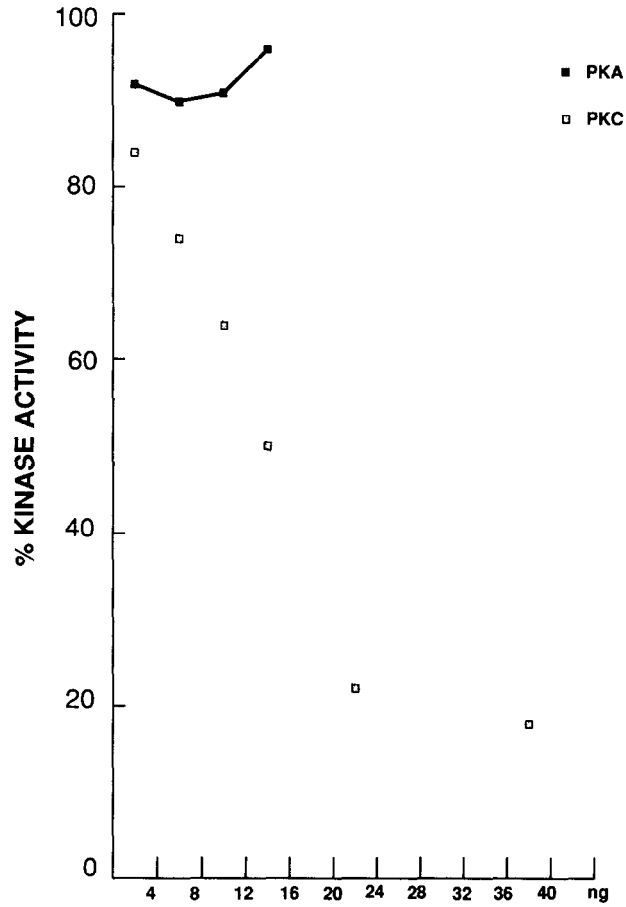

Fig. 2. Effect of increasing concentrations of IN on PKA and PKC activities. The DEAE-cellulose semipurified PKC, and the IN preparation after P11-cellulose phosphate chromatography were employed. Data are expressed as % of control PKC activity, and are the mean of three experiments.

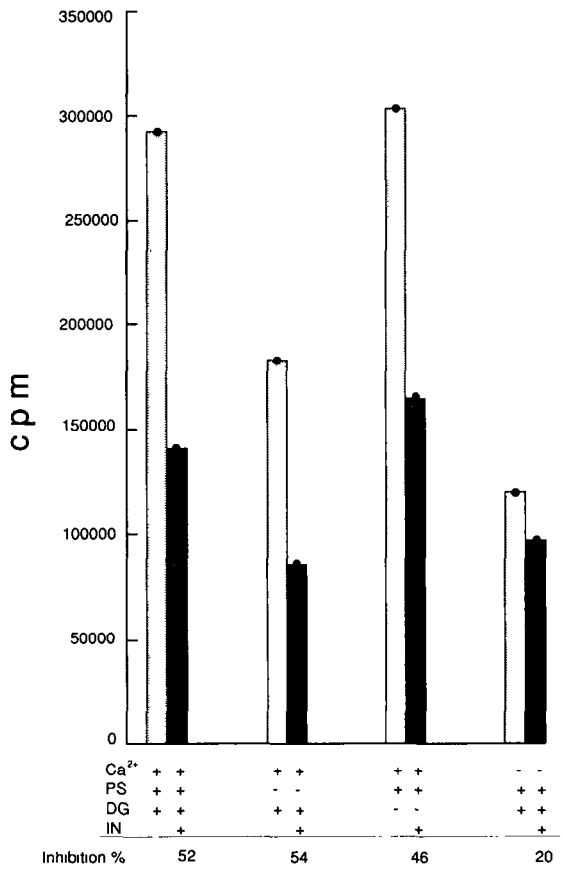

Fig. 3. Effects of different cofactors on PKC activity in the presence of IN (8–12 ng protein). The data shown are the mean of three experiments. The same preparations of PKC and IN as specified in Fig. 2 were employed.

strate histone III S up to 3× (1.5 mg/ml) did not modify the degree of IN inhibition of PKC activity in vitro. When IN was added to PHA-activated PBMC cultures particulate PKC activity was significantly reduced. However, particulate PKC activity in TPA-activated PBMC was unaffected.

4. DISCUSSION

Our data demonstrate that the SptMF of stimulated human PBMC contains a factor capable of inhibiting PKC activity 'in vitro' and 'in vivo' (IN). IN is not active against the c-AMP-dependent protein kinase indicating that the inhibitory effect is specific and not due to the presence of unidentified charged compounds that may exert an inhibitory action on PKC translocated to the membrane [1]. The IN is eluted from PC11 at an NaCl concentration of 1.35–1.50 in an oligomeric form; as demonstrated by SDS-gel electrophoresis, β-MSH in the sample buffer cleaves the native form in lower molecular weight subunits. The native form is less efficient than the subunits as inhibitor of cytosolic PKC activity, though it is completely efficient on particulate PKC. Disulfide bridges present in IN raise the question of whether these groups form disulfide bridges with the cysteine-rich region of the regulatory domain of PKC.

IN does not belong to the class of pseudosubstrate peptides, as it is not active on the enzyme's catalytic site and it is specific for PKC [16]. At least four sets of data are in favour of a specific interaction of IN with the enzyme's regulatory domain after Ca^{2+} binding: (1) the lack of inhibition of the activity generated by mild tryptic hydrolysis of PKC without Ca^{2+} and PL; (2) unsuccessful inhibition of protamine phosphorylation by PKC; (3) the failure to inhibit the activity of PKC translocated to the membrane by TPA; (4) the permissive role of Ca^{2+} binding to the regulatory domain for the IN induced PKC inhibition.

Preparation of IN from resting PBMC has been unsuccessful. Cycloheximide treatment (15 μg/ml) [14] has no effect. The finding suggests that IN is constitutively preformed in PBMC and that PHA treatment induces post-translational modification that activates IN. IN is stabilized by heating. The prolonged heating step (45 min) was found necessary to remove other heat stable protein contaminants from the preparations. The very high stability of the peptide during heating could be explained on the basis of its low molecular mass which probably allows a rapid recovery of the active form of the protein molecule.

A number of PKC inhibitors have been reported in the literature, but none of them has properties and char-

Table III

Effect of IN on the activity of PKC under different conditions: (A) in the presence of histone III S or protamine as PKC substrates; (B) after mild tryptic digestion of PKC

A

Substrate	PKC (U)		
	Without IN	With IN	Inhibition (%)
Histone III S	1.86	1.17	37
Protamine (0.01 ng/ml)	0.20	0.17	–

B

Treatment	PKC (U)		
	Before tryptic digestion	After tryptic digestion	
		Plus Ca^{2+} and PL	Minus Ca^{2+} and PL
Control	0.63	0.12	0.08
IN	0.40 (inhibition = 37%)		0.08 (inhibition = 0)

The DEAE-cellulose semipurified PKC and the IN preparation after P11-cellulose phosphate chromatography were employed. The data shown correspond to the highest PKC activity detected. The data shown are the mean of three experiments. For details see section 2.

acteristics comparable to those of IN [15–24]. IN is a constitutive peptide found in microsomal vesicles. Presumably it may become available in other cellular compartments and in the cytosol can exert its effect on the regulatory domain of translocated PKC, on the cytosolic side of the membrane lipid bilayer. IN can penetrate the cell membrane and reach the cytosolic face of the membrane, but the mechanism of penetration across the cell membrane remains to be investigated. IN does not prevent PKC activation and it becomes fully effective only after the binding of Ca^{2+} to the regulatory domain. IN appears therefore to be a true 'physiological modulator' of PKC activity.

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REFERENCES

[1] Epand, R.M. and Lester, D.S. (1990) Trends Pharmacol. Sci. 11, 317–320.
[2] Nishizuka, Y. (1988) Nature 334, 661–665.
[3] Asaoka, Y., Nakamura, S., Yoshida, K. and Nishizuka, Y. (1992) Trends Biochem. Sci. 17, 414–417.
[4] Stabel, S. and Parker, P.J. (1991) Pharmac. Ther. 51, 71–95.
[5] Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97), 77–83.
[6] Poltronieri, L., Melloni, E., Rubini, M., Selvatici, R., Mazzilli, M.C., Baricordi, O.R. and Gandini, E. (1988) Biochem. Biophys. Res. Commun. 156, 46–51.
[7] Orlando, P., Selvatici, R., Rubini, M., Balboni, A. and Gandini, E. (1990) Biochem. Int. 22, 119–123.
[8] Selvatici, R., Orlando, P., Rubini, M., Balboni, A., Balugani, S. and Gandini, E. (1991) Biochem. Int. 23, 53–56.
[9] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[10] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Cakiroglu, A.G., Jackson, J.F., Rifkind, R.A. and Marks, P.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5282–5286.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press.
[13] Bazzi, M.D. and Nelsestuen, G.L. (1987) Biochemistry 26, 1974–1982.
[14] Reed, J.C., Alpers, J.D., Nowell, P.C. and Hoower, G.R. (1986) Proc. Natl. Acad. Sci. USA 83, 3982–3986.
[15] Isakov, N., Mally, M.I., Scholz, W. and Altman, M. (1987) Immunol. Rev. 95, 89–103.
[16] Smith, M.K., Colbran, R.J. and Soderling, T.R. (1990) J. Biol. Chem. 265, 1837–1840.
[17] Hidaka, H.M., Kawamoto, I.S. and Ssaki, Y. (1986) Biochemistry 23, 5036–5041.
[18] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[19] Raynor, R.L., Zheng, B. and Kuo, J.F. (1991) J. Biol. Chem. 266, 2753–2758.
[20] Hannun, Y.A., Loomis, C.R., Merrill, A.H. and Bell, R.M. (1986) J. Biol. Chem. 261, 12604–12609.
[21] Jefferson, A.B. and Schulman, H. (1988) J. Biol. Chem. 263, 15241–15244.
[22] Hannun, Y.A. and Bell, R.M. (1988) J. Biol. Chem. 263, 5124–5131.
[23] Sullivan, J.P., Connor, J.R., Shearer, B.G. and Burch, R.M. (1991) Mol. Pharm. 41, 38–44.
[24] Balazovich, K.J., McEwen, E.L., Lutzke, M.L., Boxer, L.A. and White, T. (1992) Biochem. J. 284, 399–405.
[25] Toker, A., Sellers, L.A., Amess, B., Pately, A., Harrisa and Aitken, A. (1992) Eur. J. Biochem. 206, 453–451.