Volume 331, number 3, 267–271 FEBS 13050 October 1993

A role for protein kinase C-ε in angiotensin II stimulation of phospholipase D in rat renal mesangial cells

Josef Pfeilschifter*, Andrea Huwiler

Department of Pharmacology, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Received 22 July 1993

The role of Ca^{2+} and protein kinase C (PKC) in the regulation of phosphatidylcholine-hydrolyzing phospholipase D (PLD) was investigated in angiotensin II-stimulated mesangial cells. Elevation of cytosolic free Ca^{2+} by the calcium ionophore, A23187, or the Ca^{2+}-ATPase inhibitor, thapsigargin, slightly increased PLD-stimulated phosphatidylethanol formation. However, chelation of cytosolic Ca^{2+} with high concentrations of quin 2 did not attenuate angiotensin II-induced phosphatidylethanol production, thus suggesting that Ca^{2+} is not crucially involved in agonist-stimulated PLD activation. Stimulation of PKC by phorbol esters increased PLD activity in mesangial cells. Down-regulation of PKC-α and -δ isoenzymes by 8 h phorbol ester treatment still resulted in full PLD activation. In contrast, a 24 h treatment of mesangial cells with phorbol ester, a regimen that also causes depletion of PKC-ε, abolished angiotensin II-evoked phosphatidylethanol formation. In addition, the selective PKC inhibitor, calphostin C, attenuated hormone-induced PLD activity. In summary, these data suggest that angiotensin II stimulation of phospholipase D appears to involve the PKC-ε isoenzyme, activated by DAG derived from phosphoinositide hydrolysis.

Angiotensin II; Phospholipase D; Protein kinase C; Isoenzyme; Mesangial cell

1. INTRODUCTION

Mesangial cells are an important determinant in the regulation of the glomerular filtration rate. Morphologically, mesangial cells resemble vascular smooth muscle cells, able to contract upon stimulation by vasoactive hormones like angiotensin II [1]. Angiotensin II binds to specific AT_1 receptors on the surface of mesangial cells and triggers the rapid generation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (DAG), which are concomitantly formed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate [1,2]. Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from intracellular storage sites and DAG activates protein kinase C (PKC). These biochemical signals are thought to interact to cause a wide variety of cellular responses [3,4].

Another important signalling pathway appears to act exclusively on phosphatidylcholine in response to a wide variety of agonists and thus generates phosphatidic acid and DAG from lipid sources other than phosphoinositides [5,6]. In mesangial cells, angiotensin II [7], platelet-derived growth factor [8], endothelin [9], vasopressin [10] and extracellular nucleotides [11] have been reported to stimulate phosphatidylcholine hydrolysis by phospholipase D activity.

In the present study we addressed the question whether phospholipase D activation is dependent upon prior phosphoinositide hydrolysis, Ca^{2+} mobilization and protein kinase C activation.

*Corresponding author. Fax: (41) (61) 267 2208.

Abbreviations: PKC, protein kinase C; DAG, 1,2-diacylglycerol; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PDD, 4α-phorbol 12,13-didecanoate; PDBu, phorbol 12,13-dibutyrate.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[^{3}H]Myristic acid was purchased from Amersham International, UK; cell culture media and nutrients were from Gibco BRL, Basel, Switzerland; calphostin C, K252a, CGP 41251 and CGP 42700 were from Ciba-Geigy Ltd., Basel, Switzerland; angiotensin II, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (PDD), and phorbol 12,13-dibutyrate (PDBu) were from Calbiochem, Lucerne, Switzerland. TLC plates and all other chemicals used were either from Merck, Darmstadt, Germany, or from Fluka, Buchs, Switzerland.

2.2. *Cell culture*

Rat glomerular mesangial cells were cultured as described previously [12]. In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments, desmin and vimentin, which are considered to be specific for myogenic cells [13], positive staining for Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin-excluded endothelial and epithelial contaminations, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line [2]. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and bovine insulin at 0.66 U/ml (Sigma).

2.3. *Determination of [^{3}H]phosphatidylethanol production*

Angiotensin II-stimulated phosphatidylcholine hydrolysis was measured in cells pre-labelled for 24 h with [^{3}H]myristic acid (2.0

μCi/ml; specific activity 54 Ci/mmol). Pre-labelled confluent cells were washed several times with medium to remove unincorporated label. The cells were incubated for a further 1 h. Ethanol was added to cell monolayers 5 min prior to the addition of angiotensin II. Incubations were terminated by collecting the medium and adding ice-cold methanol to the cells. After harvesting the cells from the dishes with a rubber policeman, lipids were extracted according to Bligh and Dyer [13]. Radioactivity in aliquots of medium, aqueous phase and chloroform phase was determined. Lipid extracts were separated by thin-layer chromatography, developed with the top phase of ethyl acetate/2,2,4-trimethyl pentane/acetic acid/water (13:2:3:10, by vol.) for separation of phosphatidylethanol.

2.4. *Statistical analysis*

Statistical analysis was performed by one-way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons, according to Bonferroni.

3. RESULTS AND DISCUSSION

We have previously shown that angiotensin II triggers a delayed but sustained activation of phosphatidylcholine-degrading PLD activity in mesangial cells. The potent inhibitory action of Dup 753 and the ineffectiveness of CGP 42112A on angiotensin II-stimulated choline and phosphatidylethanol formation, as well as the rank order of potency of angiotensin II, angiotensin(1-7) and angiotensin(1-6) clearly demonstrated that this action is due to angiotensin II AT_1 receptor activation [7]. The mechanism by which hormones such as angiotensin II activate PLD is still under debate. There is substantial evidence that PLD stimulation is secondary to phosphoinositide hydrolysis and is mediated to varying degrees by Ca^{2+} and PKC [3,4]. However, other investigations have implicated G-proteins as direct activators of PLD [3,4]. In an attempt to address this issue in mesangial cells we were, therefore, interested to investigate what role, if any, the increased intracellular Ca^{2+} and PKC play in angiotensin II-stimulated phospholipase D-mediated phosphatidylcholine hydrolysis. The requirement of Ca^{2+} for phospholipase D activity was examined using the Ca^{2+} ionophore, A 23187, the Ca^{2+} ATPase inhibitor, thapsigargin, and by chelation of intracellular Ca^{2+} with high concentrations of quin 2. As shown in Fig. 1, A23187 (1 μM) and thapsigargin (200 nM) slightly increased phosphatidylethanol formation. However, chelation of cytosolic Ca^{2+} with high doses of the Ca^{2+} buffer quin 2 did not attenuate angiotensin II-stimulated phosphatidylethanol formation (Fig. 1), thus suggesting that an increase in cytosolic free Ca^{2+} is not essential for phospholipase D activation by nucleotides in mesangial cells. In this connection it is noteworthy that endothelin-stimulated phospholipase D was found to be Ca^{2+}-independent in mesangial cells [9].

The possible involvement of PKC in hormone stimulation of phospholipase D activity was examined using phorbol esters, PKC down-regulation experiments, as well as a specific inhibitor of PKC. As shown in Fig. 2 the PKC activators PMA and PDBu caused a marked increase in phosphatidylethanol production. In contrast, the biologically inactive phorbol ester, PDD, did not activate phospholipase D (Fig. 2). Furthermore, PKC down-regulation by prolonged PMA treatment (24 h) blocked angiotensin-II stimulated phosphatidylethanol synthesis (Fig. 3). Stimulation of PLD is almost a universal cellular response to phorbol ester treatment [3,4]. However, the mechanism by which phorbol esters activate PLD is not known. It is well established that PMA and PDBu bind to and activate PKC, but other cellular substrates have also been described. In fact, a recent study provided evidence that PKC may activate

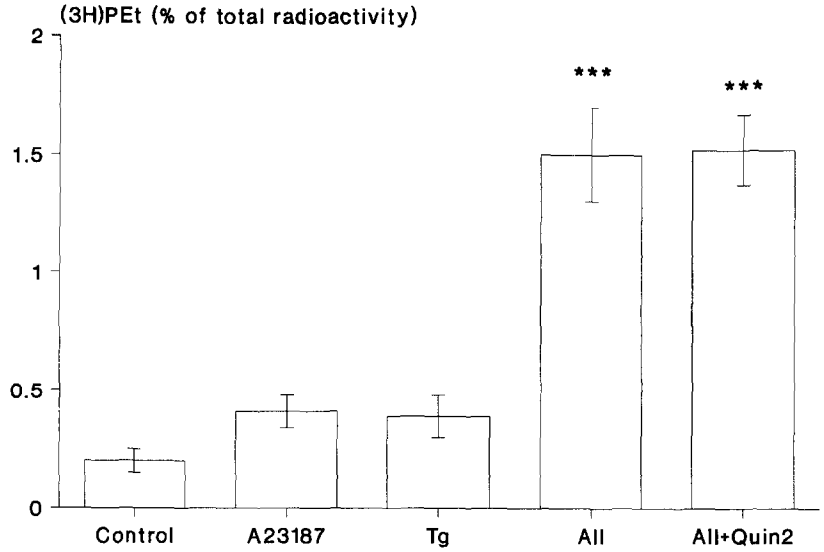

Fig. 1. Effects of Ca^{2+} on phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]myristic acid and pretreated for 60 min with 100 μM quin 2/AM where indicated. Cultures were then stimulated with angiotensin II (AII, 100 nM), the Ca^{2+} ionophore, A23187 (1 μM), or the Ca^{2+}-ATPase inhibitor, thapsigargin (Tg, 200 nM), for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in section 2. Results are means ± S.D. of four experiments. Significant differences from control: ***$P < 0.001$; ANOVA.

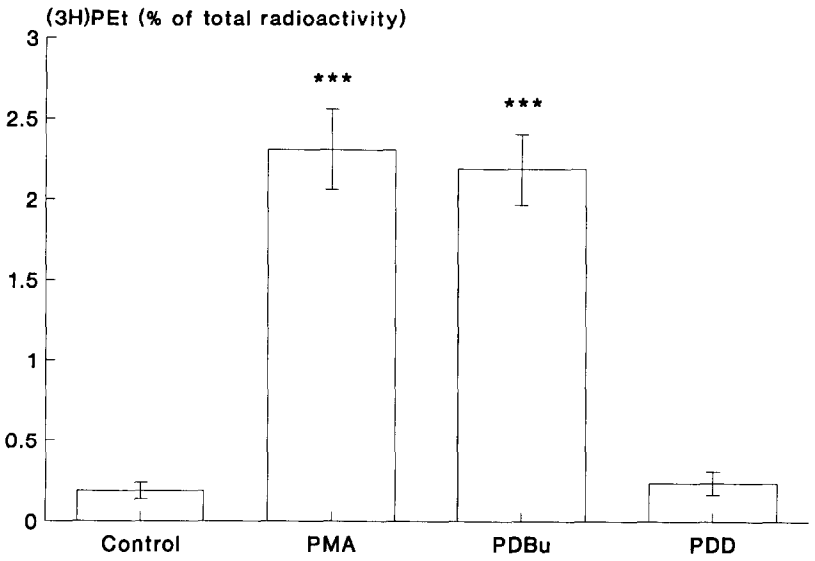

Fig. 2. Phorbol ester-stimulation of phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]myristic acid and then stimulated with PMA (100 nM), PDBu (100 nM) or PDD (100 nM) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in section 2. Results are means ± S.D. of four experiments. Significant differences from control: ***$P < 0.001$; ANOVA.

PLD by a phosphorylation-independent event [14]. Moreover, molecular cloning has revealed that PKC exists as a family of at least ten molecular species, all having closely related structures but differing in tissue and cellular distribution and in their individual enzymological characteristics [3]. By immunoblot analysis we have shown previously that mesangial cells express 4 PKC isoenzymes, PKC-α, -δ, -ε and -ζ. No PKC-β, -γ and -η isoforms were detected [15–18]. On exposure to PMA, these isoenzymes displayed distinctly different down-regulation kinetics. An 8 h treatment with PMA was sufficient to completely deplete mesangial cells of PKC-α and -δ isotypes, and a 24 h incubation with PMA was necessary to down-regulate PKC-ε. In contrast, PMA treatment for 24 h did not include any depletion of PKC-ζ [15–18]. By comparing the down-regulation kinetics of the mesangial cell PKC isoenzymes with the time-course of removal of the specific cellular func-

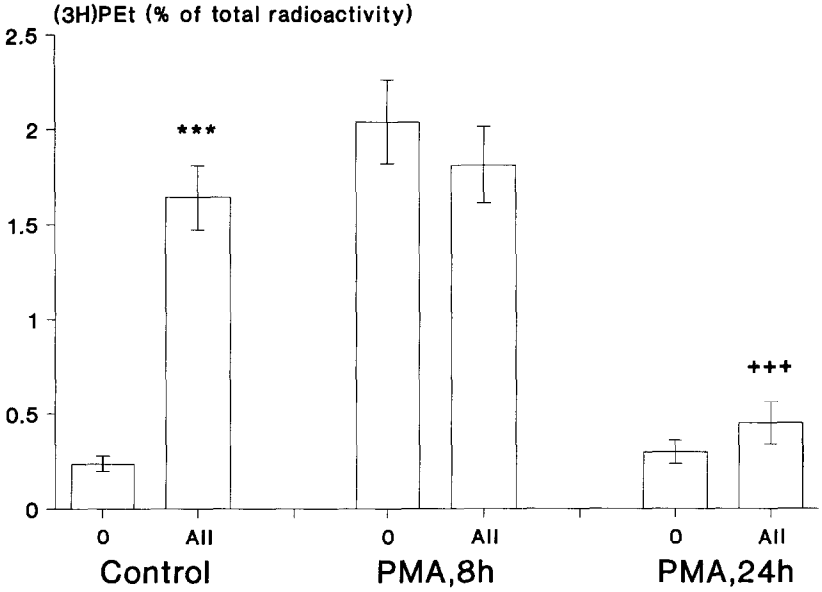

Fig. 3. Effects of PKC down-regulation on angiotensin II-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]myristic acid and either non-pretreated (control) or pretreated for 8 h or 24 h with PMA (500 nM) as indicated. Cultures were then washed and stimulated with angiotensin II (AII, 100 μM) or vehicle (O) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in section 2. Results are means ± S.D. of four experiments. Significant differences from corresponding control (without stimulation): ***$P < 0.001$; ANOVA. Significant differences from corresponding control angiotensin II stimulation (without PMA pretreatment): $^{+++}P < 0.001$; ANOVA.

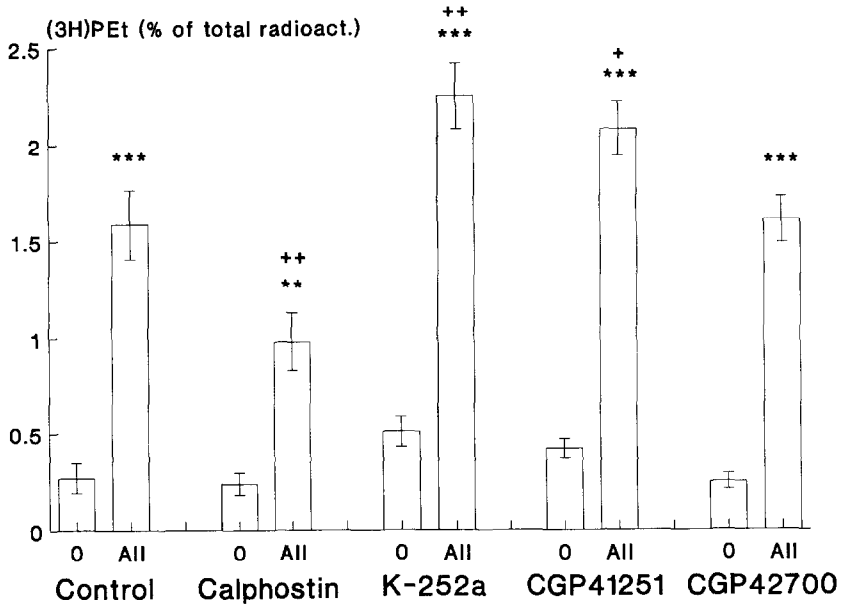

Fig. 4. Effects of PKC inhibitors on angiotensin II-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]myristic acid and pretreated for 30 min with calphostin C (1 μM), K-252a (250 nM), CGP 41251 (250 nM), CGP 42700 (250 nM) or vehicle (Control). Cultures were then stimulated with angiotensin II (AII, 100 nM) or vehicle (O) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in section 2. Results are means ± S.D. of four experiments. Significant differences from corresponding control: $^{***}P < 0.001$; $^{**}P < 0.01$; ANOVA. Significant differences from corresponding angiotensin II stimulation in the absence of inhibitor: $^{++}P < 0.01$; $^{+}P < 0.05$, ANOVA.

tions, we have suggested that PKC-α may negatively regulate phosphoinositide turnover in response to angiotensin II [15–17,19], whereas PKC-ε is a candidate for regulation of phospholipase A_2 and prostaglandin synthesis in mesangial cells [15]. Using this type of down-regulation regimen to differentially deplete PKC isoenzymes from mesangial cells we wanted to address the question which of the four isoforms present in the cells is involved in regulating PLD activity. An 8 h treatment with PMA that causes depletion of α- and δ-isoenzymes of PKC in mesangial cells [15,16] was not sufficient to eliminate angiotensin II-induced phospholipase D activation (Fig. 3). It was necessary to preincubate the cells for 24 h with PMA, a regimen that also causes depletion of PKC-ε [15], to fully prevent the responses to angiotensin II (Fig. 3). Moreover, the selective PKC inhibitor, calphostin C (1 μM), which interacts with the regulatory domain of the enzyme [20], attenuated angiotensin II-stimulated phosphatidylethanol formation (Fig. 4). In summary, these data suggest that PKC-ε may not only activate phospholipase A_2 in mesangial cells, but also phosphatidylcholine-specific PLD.

In this connection it is worth noting that inhibitors of PKC which display a selectivity for the Ca^{2+}-dependent group A isoenzymes, as compared to the Ca^{2+}-independent group B isotypes, such as staurosporine, K-252a or the specific PKC inhibitor, CGP 41251 ([21–23], Mate, B.M., Meyer, T., Stabel, S., Jaken, S., Fabbro, D. and Hynes, N.E., manuscript submitted), did not inhibit angiotensin II-induced phospholipase D activation (Fig. 4). In contrast, K-252a and CGP41251 even potentiated angiotensin II-induced PLD activity (Fig. 4). A possible explanation for this observation may be the potent inhibition of the Ca^{2+}-dependent PKC-α by these inhibitors. As PKC-α mediates the feedback inhibition of hormone-stimulated phosphoinositide turnover in mesangial cells [15,16], pretreatment of cells with the inhibitors results in an augmentation of inositol trisphosphate and DAG formation in response to angiotensin II [19]. The increased generation of DAG may cause a pronounced activation of PKC isotypes, including PKC-ε, and thereby potentiate PLD activity. As a control, we have also included CGP 42700, a staurosporine derivative that is devoid of any PKC inhibitory activity [23]. Fig. 4 shows that CGP 42700 did not alter basal or hormone-induced formation of phosphatidylethanol. Failure to achieve inhibition of PLD with PKC inhibitors has led to the suggestion that phorbol ester may act, in part, through a PKC-independent mechanism [5,6]. Our observation could provide an alternative explanation for some of these reports. However, one should be aware that there may be a cell type-specific link between certain PKC isoenzymes and PLD activation. Over-expression of PKCβ_1 in fibroblasts has been reported to enhance PLD activity after PMA or endothelin-1 stimulation [24,25]. Furthermore, over-expression of PKC-α in Swiss 3T3 fibroblasts upregulates PLD, leading to a constitutive higher level of enzyme activity [26]. On the other hand, DAG derived

from PLD-mediated phosphatidylcholine hydrolysis has been shown to selectively activate PKC-β in interferon-α-activated leucocytes [27] and PKC-ζ in activated *Xenopus* oocytes [28]. Thus, PKC-mediated activation of PLD, and the subsequent generation of DAG, may provide a positive-feedback loop to sustain PKC activation in cells. Whether phosphatidylcholine hydrolysis, prolonged formation of DAG and sustained activation of PKC are a common response to mitogenic signals that crucially function in mitogenic signal transduction, as recently suggested by Cook and Wakelam [29], remains to be elucidated.

Acknowledgements: This work was supported by a grant from Ciba-Geigy Ltd. for A.H. We gratefully acknowledge supply of PKC inhibitors by Dr. G. Caravatti and Dr. T. Meyer, Ciba-Geigy Ltd., Basel, Switzerland.

REFERENCES

[1] Pfeilschifter, J. (1989) Eur. J. Clin. Invest. 19, 347–361.
[2] Pfeilschifter, J. (1990) Eur. J. Pharmacol. 184, 201–202.
[3] Nishizuka, Y. (1992) Science 258, 607–614.
[4] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–204.
[5] Billah, M.M. and Anthes, J.C. (1990) Biochem. J. 269, 281–291.
[6] Exton, J.H. (1990) J. Biol. Chem. 265, 1–4.
[7] Pfeilschifter, J., Huwiler, A., Merriweather, C. and Briner, V.A. (1992) Eur. J. Pharmacol. Mol. Pharmacol. 255, 57–62.
[8] Pfeilschifter, J. and Hosang, M. (1991) Cell Signal. 3, 413–424.
[9] Kester, M., Simonson, M.S., McDermott, R.G., Baldi, E. and Dunn, M.J. (1992) J. Cell. Physiol. 150, 578–585.
[10] Troyer, D.A., Gonzalez, O.F., Padilla, R.M. and Kreisberg, J.I. (1992) Am. J. Physiol. 262, F185–F191.
[11] Pfeilschifter, J. and Merriweather, C. (1993) Br. J. Pharmacol. (in press).
[12] Pfeilschifter, J., Kurtz, A. and Bauer, C. (1984) Biochem. J. 223, 855–859.
[13] Travo, P., Weber, K. and Osborn, M. (1982) Exp. Cell. Res. 139, 87–94.
[14] Conricode, K.M., Brewer, K.A. and Exton, J.H. (1992) J. Biol. Chem. 267, 7199–7202.
[15] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. J. 279, 441–445.
[16] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. Biophys. Res. Commun. 180, 1422–1428.
[17] Huwiler, A., Fabbro, D., Stabel, S. and Pfeilschifter, J. (1992) FEBS Lett. 300, 259–262.
[18] Huwiler, A., Schulze-Lohoff, E., Fabbro, D. and Pfeilschifter, J. (1993) Exp. Nephrol. 1, 19–25.
[19] Ochsner, M., Huwiler, A., Fleck, T. and Pfeilschifter, J. (1993) Eur. J. Pharmacol. Mol. Pharmacol. 245, 15–21.
[20] Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. 156, 548–553.
[21] Gschwendt, M., Leibersperger, H. and Marks, F. (1989) Biochem. Biophys. Res. Commun. 164, 974–982.
[22] McGlynn, E., Liebetanz, J., Reutener, S., Wood, J., Lydon, N.B., Hofstetter, H., Vanek, M., Meyer, T. and Fabbro, D. (1992) J. Cell. Biochem. 49, 239–250.
[23] Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rösel, J., Müller, M., Caravatti, G. and Matter, A. (1989) Int. J. Cancer 43, 851–856.
[24] Pai, J.-K., Pachter, J.A., Weinstein, I.B. and Bishop, W.R. (1991) Proc. Natl. Acad. Sci. USA 88, 598–602.
[25] Pai, J.-K., Dobek, E.A. and Bishop, W.R. (1991) Cell Regul. 2, 897–903.
[26] Eldar, H., Pen-Av, P., Schmidt, U.-S., Livneh, E. and Liscovitch, M. (1993) J. Biol. Chem. 268, 12560–12564.
[27] Pfeffer, L.M., Strulovici, B. and Saltiel, A.R. (1990) Proc. Natl. Acad. Sci. USA 87, 6537–6541.
[28] Dominguez, I., Diaz-Meco, M.T., Municio, M.M., Berra, E., Garcia de Herreros, A., Cornet, M.E., Sanz, L. and Moscat, J. (1992) Mol. Cell. Biol. 12, 3776–3783.
[29] Cook, S.J. and Wakelam, M.J.O. (1991) Cell. Signal. 3, 273–282.