Possible regulatory functions of protein kinase C- α and - ϵ isoenzymes in rat renal mesangial cells

Stimulation of prostaglandin synthesis and feedback inhibition of angiotensin II-stimulated phosphoinositide hydrolysis

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Short-term treatment of mesangial cells with phorbol 12-myristate 13-acetate (PMA) decreases angiotensin II-induced InsP_3 formation, but potentiates hormone-stimulated arachidonic acid release and prostaglandin (PG) E_2 synthesis. Long-term treatment with PMA augments hormone-stimulated InsP_3 generation (after 8 h treatment), but eliminates angiotensin II-induced arachidonic acid release and PGE_2 formation (after 24 h treatment). By using specific antibodies it is observed that mesangial cells express two protein kinase C (PKC) isoenzymes, PKC- α and - ϵ . No PKC- β and - γ isoenzymes are detected. On exposure to PMA a complete depletion of PKC- α is observed within 8 h. In contrast, down-regulation of PKC- ϵ to 10–20 % of that found in control cells requires a 24 h treatment with PMA. These results may imply that PKC- α mediates feedback inhibition of phosphoinositide hydrolysis, whereas PKC- ϵ is a candidate for regulating PG synthesis in mesangial cells.

INTRODUCTION

PKC is a family of at least seven closely related isotypes that are activated by hormone-stimulated inositol lipid turnover and participate in the signalling process by phosphorylating certain target proteins (for reviews see [1,2]). In glomerular mesangial cells, PKC exerts two major functions: it participates in the regulation of hormone-stimulated PG synthesis, and it acts as a negative-feedback regulator of the inositol lipid signalling cascade (for reviews see [3,4]). Hormones, such as angiotensin II or vasopressin, trigger a rapid formation of InsP_3 and 1,2-diacylglycerol (DG) by stimulating phospholipase C-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. This bifurcating signalling cascade provides an elegant mechanism of auto-regulation, in that PKC functions as a negative-feedback regulator of phospholipase C activity [3]. Treatment of mesangial cells with PKC activators such as PMA inhibits angiotensin II-, vasopressin- and ATP-stimulated generation of InsP_3 and subsequent Ca^{2+} mobilization [5–7]. Furthermore, inhibitors of PKC, such as H-7, sphingosine or cytochalasin I, augment angiotensin II-stimulated InsP_3 formation [8]. In addition, in PKC-depleted mesangial cells a potentiation of angiotensin II-induced InsP_3 generation and Ca^{2+} is observed [9]. These results are in agreement with the idea that blockage or down-regulation of PKC would result in an increased responsiveness of the phosphoinositide pathway to hormone stimulation. Phorbol esters which activate PKC are capable of stimulating PG synthesis in mesangial cells [6,8,10]. Inhibitors of PKC suppress the stimulatory effect of PMA on PG synthesis, suggesting a role for PKC in the regulation of eicosanoid synthesis [8]. Furthermore, a synergistic action of Ca^{2+} and PMA on phospholipase A_2 activity is observed in mesangial-cell arachidonic acid release and PG synthesis [11]. The objective of the present work was to identify the isoenzymes of PKC present in mesangial cells and to compare their relative susceptibility to

down-regulation with the down-regulation of the functional cell responses, i.e. stimulation of PG synthesis and feedback inhibition of InsP_3 generation.

MATERIALS AND METHODS

Chemicals

Angiotensin II, PMA and 4 α -phorbol 12,13-didecanoate were purchased from Calbiochem, Lucerne, Switzerland; *myo*-[2- ^3H]inositol was purchased from Amersham International, Amersham, Bucks., U.K.; [^3H]arachidonic acid (sp. radioactivity 240 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; all cell-culture nutrients were from Boehringer, Mannheim, Germany; all other chemicals used were from either Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

Cell culture

Rat renal mesangial cells were cultivated as described previously [10]. In a second step, single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial-cell morphology were used for further processing. The cells were grown in RPMI 1640 supplemented with 10 % (v/v) fetal-calf serum, penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and bovine insulin (0.66 unit/ml). Mesangial cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells [12], positive staining for Thy 1.1 antigen, and negative staining for factor-VIII-related antigen and cytokeratin, which excludes endothelial and epithelial contaminations. Generation of InsP_3 upon activation of angiotensin II B-type receptor [13] was used as a functional criterion for characterizing the cloned cell line. For these experiments, passages 9–28 of mesangial cells were used.

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PG, prostaglandin; DG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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Fig. 1. Time course of PMA effects on angiotensin II-stimulated InsP_3 formation (a), arachidonic acid release (b) and PGE_2 synthesis (c) in mesangial cells

Cells were labelled with *myo*-[^3H]inositol (a) or [^3H]arachidonic acid (b), treated with PMA (500 nM) for the indicated time periods and then exposed to angiotensin II (100 nM) for 20 s (a) or 30 min (b and c). InsP_3 (a), arachidonic acid (AA) release (b) and PGE_2 synthesis (c) were determined as described in the Materials and methods section. Results are expressed as percentage of the respective control values obtained from angiotensin II-stimulated control cells. Results are means $\pm$ S.E.M. ($n = 4$).

Determination of inositol phosphates

Confluent cells in 35 mm-diameter dishes were labelled for 72 h with *myo*-[2- ^3H]inositol (10 $\mu\text{Ci}/\text{ml}$) in RPMI 1640 medium free of inositol, containing 2% dialysed fetal-calf serum. Phorbol esters or vehicle were added, when not otherwise indicated, for the last 24 h of the labelling period. Thereafter, the medium was removed and the cells were rinsed several times to remove free [^3H]inositol and incubated for an additional 1 h in fresh medium. After this procedure, mesangial cells were incubated in 1 ml of Dulbecco's modified Eagle's medium (DMEM) with or without angiotensin II for the indicated time periods. Thereafter, the reaction was terminated by rapid aspiration of the medium and addition of 1 ml of 15% (w/v) trichloroacetic acid. For extraction of inositol phosphates the dishes were put on ice for 1 h. The trichloroacetic acid was then removed with diethyl ether and the final extract was neutralized and applied to anion-exchange columns containing 1 ml of Dowex 1-X8 (100–200 mesh, formate form; Serva, Heidelberg, Germany). Free inositol and the inositol phosphates were eluted sequentially in accordance with Berridge [14] as described previously [10]. As observed previously, long-term treatment of mesangial cells with PMA decreased the total water-soluble [^3H]inositol content of the cells [9]. Since water-soluble [^3H]inositol and its phosphorylated metabolites were co-processed in the separation assay, and thus should be subject to

the same losses, the ratios of the phosphorylated compounds relative to total water-soluble [^3H]inositol were calculated for each sample.

Determination of arachidonic acid release

Confluent cells in 16 mm-diameter wells were labelled for 48 h with [^3H]arachidonic acid (1 $\mu\text{Ci}/\text{ml}$; sp. radioactivity 240 Ci/mmol) in DMEM, containing 0.1 mg of fatty-acid-free BSA/ml. When not otherwise indicated, phorbol esters or vehicle were added for the last 24 h of the labelling period. Thereafter, the medium was sampled for remaining radioactivity and the cells were washed three times to remove all unincorporated [^3H]arachidonic acid. Approx. 80–90% of the added [^3H]arachidonic acid was incorporated by this procedure. The labelled cells were incubated in DMEM containing 1 mg of BSA/ml as a trap for the released [^3H]arachidonic acid. The cells were then stimulated with angiotensin II or vehicle for the indicated time periods. Thereafter the medium was removed and centrifuged. Cells were dissolved in 0.5 M-NaOH, and radioactivity was counted in the supernatants and cell extracts in a scintillation counter. The percentage of [^3H]arachidonic acid released from total incorporated radioactivity was calculated.

Determination of PGE_2 synthesis

Confluent cells in 16 mm-diameter wells were washed and incubated for 48 h in DMEM, containing fatty-acid-free BSA (0.1 mg/ml). When not otherwise indicated, phorbol esters or vehicle were added, for the last 24 h of the incubation period. Thereafter the cells were washed and stimulated with angiotensin II or vehicle for 30 min. At the end of an experiment, the medium was withdrawn and centrifuged. The supernatant was assayed for PGE_2 by radioimmunoassay (New England Nuclear). Cells were dissolved in 0.5 M-NaOH and protein was determined by the method of Lowry *et al.* [15], with BSA as a standard.

Peptide synthesis and generation of antibodies

A synthetic peptide based on the C-terminal sequence of PKC- ϵ (NQEEFKGFSYFGEDLMP) was synthesized on an ARI 431 peptide synthesizer, coupled to keyhole-limpet haemocyanin by glutaraldehyde and used to immunize rabbits as described previously [16]. Antibodies were purified by using the PKC- ϵ peptide coupled to epoxy-activated Sepharose 6B. The specificity of PKC- ϵ antiserum has been studied (D. Fabbro, T. Meyer & S. Stabel, unpublished work).

Immunodetection of PKC

Confluent mesangial cells in 100 mm-diameter dishes were washed with phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/8 mM- $\text{Na}_2\text{HPO}_4/1.5$ mM- KH_2PO_4) and incubated in 10 ml of DMEM containing 0.1 mg of fatty-acid-free BSA/ml with or without phorbol esters. After the indicated time periods, the medium was removed. The cells were washed with ice-cold PBS and scraped into 0.5 ml of ice-cold homogenization buffer (20 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 1 mM-EGTA, 2 mM-dithiothreitol, 25 μg of leupeptin/ml, 30 mM-phenylmethanesulphonyl fluoride, 10 mM-benzamide) with a rubber policeman. All subsequent steps were carried out at 4°C. The cells were lysed with 3×10 s bursts with a Branson B15 sonifier (setting 4.0) and centrifuged for 1 h at 100 000 g . Supernatants were used as a source of cytosolic protein. Pellets were re-sonicated in 1 ml of the same buffer containing 1% (v/v) Triton X-100 and centrifuged for 1 h at 100 000 g , yielding the solubilized particulate fractions. Protein concentration was determined by the method of Bradford [17]. The PKC fractions were subjected to SDS/PAGE (8%-acrylamide gel) as described by Laemmli [18], and proteins were transferred on to nitrocellulose paper for

Fig. 2. Immunoblot analysis of down-regulation of PKC isoenzymes in mesangial cells

Mesangial cells were treated with PMA (500 nM) for the indicated time periods, and the cytosolic and particulate fractions were prepared as described in the Materials and methods section. Samples (70 µg of protein) were subjected to SDS/PAGE, transferred to nitrocellulose, and Western-blot analysis was performed by using either monoclonal antibodies against PKC-α at a dilution of 1:100 or antiserum against PKC-ε at a dilution of 1:1000. Bands were detected with horseradish peroxidase.

1 h at 250 mA by using a Bio-Rad Transblot apparatus. The blotting buffer used was 25 mM-Tris/HCl (pH 7.4)/190 mM-glycine in 20% (v/v) methanol. After the transfer, nitrocellulose filters were washed extensively in distilled water and blocked with 3% (w/v) BSA in PBS for 1 h at 37 °C. Filters were then incubated for 4 h at 25 °C with monoclonal antibodies raised against the PKC-α, -β and -γ isoenzymes [19] or antiserum reactive with PKC-ε (diluted in PBS containing 0.1% BSA as indicated in Fig. 2 legend). After washing in 0.1% (v/v) Tween 20 in PBS (4 × 5 min), the filters were incubated for 30 min with horseradish-peroxidase-conjugated anti-mouse IgG antibodies (PKC-α) or anti-rabbit IgG antibodies (PKC-ε), in PBS containing 0.1% BSA and 0.1% Tween 20. Finally, for colour reaction the filters were incubated in PBS containing 0.5 mg of 3,3'-diaminobenzidine/ml and 0.03% H₂O₂ for 10 min and then washed extensively in distilled water.

RESULTS

Time course of PMA effects on angiotensin II-stimulated InsP₃ formation

Stimulation of mesangial cells prelabelled with [³H]inositol by angiotensin II (100 nM) for 20 s causes marked increases in formation of InsP₁ (+40%), InsP₂ (+980%) and InsP₃ (+240%), consistent with previously published data [5,8,9]. Preincubation with PMA (500 nM) for 30 min has no significant effect on basal InsP₃ values (9200 ± 430 c.p.m./mg of protein in control cells, versus 8900 ± 470 c.p.m./mg of protein in PMA-treated cells; means ± S.E.M., *n* = 4), but potently attenuates angiotensin II-stimulated generation of InsP₃ (Fig. 1a). However, this inhibition is almost completely lost after incubating mesangial cells for 2 h with PMA (Fig. 1a). Furthermore, longer-term treatment with PMA even leads to an enhanced formation of InsP₃ in response to angiotensin II (Fig. 1a). This augmentation is maximal after 8 h treatment and remains elevated up to 48 h. The biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate has no effect on angiotensin II-induced InsP₃ generation, irrespective of the time of incubation (results not shown).

Time course of PMA effects on angiotensin II-stimulated release of arachidonic acid and PGE₂ synthesis

Following short-term treatment with PMA (500 nM, 30 min),

which itself causes a significant increase in arachidonic acid release (0.67 ± 0.09% of total incorporated radioactivity in control cells, versus 1.39 ± 0.15% in PMA-treated cells; means ± S.E.M., *n* = 4) and PGE₂ synthesis (0.52 ± 0.12 ng/mg of protein versus 1.24 ± 0.18 ng/mg of protein; means ± S.E.M., *n* = 4), the stimulatory effects of angiotensin II on arachidonic acid release (2.31 ± 0.25%) as well as on PGE₂ formation (1.78 ± 0.22 ng/mg of protein) are markedly augmented, as shown in Figs. 1(b) and 1(c). This PMA-induced potentiation of angiotensin II-stimulated arachidonic acid release and PGE₂ synthesis is time-dependent, reaching maximal values after 1 h of PMA treatment, and remains at this elevated level for at least 8 h. Longer-term incubations with PMA (24 h) cause complete inhibition of hormone-induced arachidonic acid release and PGE₂ synthesis (Figs. 1b and 1c).

PKC isoenzymes in mesangial cells

To determine the importance of PKC isoenzymes in these functional cell responses, we used isotype-specific antibodies for immuno-blotting procedures. Using monoclonal antibodies recognizing PKC-α, -β and -γ isoenzymes and an antiserum reactive with PKC-ε, we observed that mesangial cells express two PKC isoforms, PKC-α and -ε (Fig. 2). No PKC-β and -γ isoforms are detected (results not shown). Surprisingly, PKC-α and -ε-specific immunoreactivity is almost completely restricted to the particulate fractions, with little cytosolic localization. This may be a speciality of our cloned mesangial cell line, in that immunoblotting of primary mesangial-cell cultures shows a predominant cytosolic localization of PKC isoenzymes (A. Huwiler & J. Pfeilschifter, unpublished work). In mouse thymocytes as well as in clonal GH₄C₁ rat pituitary cells, PKC-α, -β and -ε isotypes are mostly in the cytosolic form [20,21]. In contrast, in a transfected Chinese-hamster-ovary cell line, PKC-β and -ε isoforms are predominantly in particulate form [20].

Time course of PMA-induced down-regulation of PKC-α and -ε

Short-term treatment with PMA (500 nM) for up to 1 h results in an increase of both membrane-bound PKC-α and -ε isotypes and a corresponding decrease in cytosolic isoenzymes, consistent with a translocation of the enzyme from the cytosolic to the particulate fraction (Fig. 2). Alternatively, the increase in

membrane-bound PKC- α after 1 h of PMA treatment could be simply due to a more condensed band on the blot. Longer-term incubation with PMA results in a progressive decrease of PKC- α levels, with a complete depletion observed after 8 h. In contrast, down-regulation of PKC- ϵ occurs at a much slower rate. After 8 h of incubation with PMA, there is still no apparent decrease in immunoreactivity, as compared with untreated control cells (Fig. 2). Even after 24 h exposure to PMA there is only partial loss of PKC- ϵ , to levels of 10–20 % of that found in control cells. No further loss of PKC- ϵ occurred over the next 24 h (Fig. 2).

DISCUSSION

We and others have shown that PMA inhibits hormone-induced generation of InsP_3 and the subsequent mobilization of Ca^{2+} in mesangial cells, smooth-muscle cells and endothelial cells, whereas it potentiates hormone-stimulated prostaglandin synthesis [5–7,22,23]. To elucidate the contribution of PKC to these dual effects of PMA, we determined the effect of long-term treatment with PMA on arachidonic acid release, PGE_2 synthesis and InsP_3 generation in response to angiotensin II stimulation. Long-term exposure of cells to phorbol esters results in down-regulation of PKC and disappearance of phorbol ester responsiveness [24,25], owing to enhanced degradation of PKC [25,26]. We have obtained evidence from PKC down-regulation experiments that stimulation of arachidonic acid release and PG synthesis and inhibition of InsP_3 generation are removed with different time courses (Fig. 1). Thus these data indicate that different isoenzymes of PKC with different sensitivities to down-regulation may be involved in these cellular responses. Distinct kinetics of down-regulation of functional responses in PMA-treated cells have also been reported for rat basophilic leukaemia cells, where the opposing effects of PMA on the IgE-dependent formation of InsP_3 and 5-hydroxytryptamine secretion are differentially down-regulated [27]. A differential down-regulation of PKC isoenzymes has been shown in several cellular systems [28–33]. We therefore sought to identify the isotypes of PKC present in mesangial cells, and to determine their down-regulation kinetics in order to find a possible correlation with the time courses of removal of the specific cellular functions, i.e. stimulation of PG synthesis and feedback inhibition on InsP_3 generation. Immunoblotting procedures demonstrate that mesangial cells express PKC- α and - ϵ isoenzymes, but not PKC- β and - γ . The main finding of the immunoblot analysis is that PKC- α is depleted faster than PKC- ϵ , and the respective kinetics correspond quite well to the kinetics of the functional responses of interest (Figs. 1 and 2). These observations suggest that PKC- α may negatively regulate InsP_3 generation, whereas PKC- ϵ may transmit activation of PG synthesis. Recently, Godson and colleagues [34] reported that activation of PKC- α is associated with arachidonic acid release in Madin–Darby canine kidney cells. This suggestion is based on the observed association of PKC- α translocation and arachidonic acid release and its inhibition in PKC- α -depleted cells. However, these authors only investigated PKC- α and - β isoenzymes, but not PKC- ϵ , and they performed a single 18 h phorbol ester down-regulation regimen. Thus there is no contradiction of our data, and the results of Godson *et al.* [34] could be equally well explained by our hypothesized functional association with PKC- ϵ isoenzyme. However, there is evidence for a cell-type-specific regulation of PKC isoenzymes, in that PKC- ϵ is resistant to down-regulation by PMA in mouse thymocytes and the promonocytic cell line U937 [20]. In contrast, in GH_4C_1 cells PKC- ϵ is completely removed after 12 h of phorbol ester treatment, whereas loss of PKC- α is much slower [21]. The cellular target of PKC action responsible for the inhibition of angiotensin II-stimulated InsP_3

generation is not precisely known. There is no alteration either in the number of cellular receptors for angiotensin II or in their affinity [5,9]. It appears that PKC alters angiotensin II-induced generation of InsP_3 by inhibiting the signal transmission via the coupling G-protein. It was observed that short-term treatment of mesangial cells with PMA does not affect guanosine 5'-[γ -thio]triphosphate-induced InsP_3 production from washed membranes of these cells [35]. However, PMA treatment strongly decreases the synergistic effect of angiotensin II on guanosine 5'-[γ -thio]triphosphate-stimulated InsP_3 formation, thus suggesting that the coupling between the receptor and the G-protein might have been impaired [35]. The hormone-induced release of arachidonic acid from membrane phospholipids is the rate-limiting step in the synthesis of eicosanoids [36]. The dominant factor regulating arachidonic acid liberation in most cells, including mesangial cells, is phospholipase A_2 [37,38]. Two types of phospholipase A_2 have been described in mesangial cells. One type is a low-molecular-mass (14 kDa) phospholipase $\text{A}_{2\text{a}}$, belonging to group II phospholipase A_2 , that is induced and secreted from mesangial cells upon stimulation with proinflammatory cytokines, such as interleukin 1 or tumour necrosis factor [39,40]. Mesangial cells contain an additional phospholipase A_2 that shows an apparent molecular mass of 60 kDa upon gel filtration [41,42] and of 110 kDa upon SDS/PAGE [43]. Most interesting is the fact that the activity of the latter enzyme can be increased by preincubation of the cells with vasopressin or PMA, indicating that this is a hormone-sensitive phospholipase A_2 and that PKC can increase its activity. The observed increase in enzyme activity may be due to direct phosphorylation of phospholipase A_2 by PKC, as suggested by phosphorylation experiments *in vitro* with the purified 110 kDa enzyme [44]. Further studies will be needed to identify the active PKC isoenzyme unequivocally, as well as the corresponding substrates.

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REFERENCES

1. Nishizuka, Y. (1988) *Nature* (London) **334**, 661–665
2. Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C. J., Schaap, D., Stabel, S. & Webster, C. (1989) *Mol. Cell. Endocrinol.* **65**, 1–11
3. Pfeilschifter, J. (1989) *Eur. J. Clin. Invest.* **19**, 347–361
4. Menè, P., Simonson, M. S. & Dunn, M. J. (1989) *Physiol. Rev.* **69**, 1347–1424
5. Pfeilschifter, J. (1986) *FEBS Lett.* **203**, 262–266
6. Troyer, D. A., Gonzalez, O. F., Douglas, J. G. & Kreisberg, J. I. (1988) *Biochem. J.* **251**, 907–912
7. Pfeilschifter, J. (1990) *Cell. Signalling* **2**, 129–138
8. Pfeilschifter, J. (1988) *Biochim. Biophys. Acta* **969**, 263–270
9. Pfeilschifter, J., Fandrey, J., Ochsner, M., Whitebread, S. & de Gasparo, M. (1990) *FEBS Lett.* **261**, 307–311
10. Pfeilschifter, J., Kurtz, A. & Bauer, C. (1986) *Biochem. J.* **234**, 125–130
11. Bonventre, J. V. & Swidler, M. (1988) *J. Clin. Invest.* **82**, 168–176
12. Travo, P., Weber, K. & Osborn, M. (1982) *Exp. Cell Res.* **139**, 87–94
13. Pfeilschifter, J. (1990) *Eur. J. Pharmacol.* **184**, 201–202
14. Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
16. Borner, C., Wyss, R., Regazzi, R., Eppenberger, U. & Fabbro, D. (1987) *Int. J. Cancer* **40**, 344–348
17. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
18. Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
19. Hidaka, H., Tanaka, T., Onoda, K., Hagiwara, M., Watanabe, M., Ohta, H., Ito, Y., Tsurudome, M. & Yoshida, T. (1988) *J. Biol. Chem.* **263**, 4523–4526

20. Strulovici, B., Daniel-Issakani, S., Baxter, G., Knopf, J., Sultzman, L., Cherwinski, H., Nestor, J., Jr., Webb, D. R. & Ransom, J. (1991) *J. Biol. Chem.* **266**, 168–173
21. Kiley, S., Schaap, D., Parker, P., Hsieh, L. L. & Jaken, S. (1990) *J. Biol. Chem.* **265**, 15704–15712
22. Chardonnes, D., Lang, U., Rossier, M. F., Capponi, A. M. & Vallotton, M. B. (1990) *J. Biol. Chem.* **265**, 10451–10457
23. Carter, T. D., Hallam, T. J. & Pearson, J. D. (1989) *Biochem. J.* **262**, 431–437
24. Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
25. Ballester, R. & Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 15194–15199
26. Young, S., Parker, P. J., Ullrich, A. & Stabel, S. (1987) *Biochem. J.* **244**, 775–779
27. Gat-Yablonski, G. & Sagi-Eisenberg, R. (1990) *Biochem. J.* **270**, 679–684
28. Ase, K., Berry, N., Kikkawa, Y., Kishimoto, A. & Nishizuka, Y. (1988) *FEBS Lett.* **236**, 396–400
29. Adams, J. C. & Gullick, W. J. (1989) *Biochem. J.* **257**, 905–911
30. Huang, F. L., Yoshida, Y., Cunha-Melo, J. R., Beaven, M. A. & Huang, K. P. (1989) *J. Biol. Chem.* **264**, 4238–4243
31. Cooper, D. R., Watson, J. E., Acevedo-Duncan, M., Pollet, R. J., Standaert, M. L. & Farese, R. V. (1989) *Biochem. Biophys. Res. Commun.* **161**, 327–334
32. Kariya, K., Kawahara, Y., Fukuzaki, H., Hagiwara, M., Hidaka, H., Fukumoto, Y. & Takai, Y. (1989) *Biochem. Biophys. Res. Commun.* **161**, 1020–1027
33. Isakov, N., McMahon, P. & Altman, A. (1990) *J. Biol. Chem.* **265**, 2091–2097
34. Godson, C., Weiss, B. A. & Insel, P. A. (1990) *J. Biol. Chem.* **265**, 8369–8372
35. Pfeilschifter, J. & Bauer, C. (1987) *Biochem. J.* **248**, 209–215
36. Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
37. Waite, M. (1987) in *The Phospholipases* (Waite, M., ed.), pp. 111–133, Plenum Publishing Corp., New York
38. Schlondorff, D., DeCandido, S. & Satriano, J. A. (1987) *Am. J. Physiol.* **253**, C113–C120
39. Pfeilschifter, J., Pignat, W., Vosbeck, K. & Märki, F. (1989) *Biochem. Biophys. Res. Commun.* **159**, 385–394
40. Schalkwijk, C., Pfeilschifter, J., Märki, F. & van den Bosch, H. (1991) *Biochem. Biophys. Res. Commun.* **174**, 268–275
41. Gronich, J. H., Bonventre, J. V. & Nemenoff, R. A. (1988) *J. Biol. Chem.* **263**, 16645–16651
42. Bonventre, J. V., Gronich, J. H. & Nemenoff, R. A. (1990) *J. Biol. Chem.* **265**, 4934–4938
43. Gronich, J. H., Bonventre, J. V. & Nemenoff, R. A. (1990) *Biochem. J.* **271**, 37–43
44. Gronich, J. H. & Nemenoff, R. A. (1990) *J. Am. Soc. Nephrol.* **1**, 470 (abstr.)

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