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Immunocharacterization of δ- and ζ-isoenzymes of protein kinase C in rat renal mesangial cells

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The isoforms of protein kinase C (PKC) present in rat mesangial cells were identified by immunoblot analysis with antibody raised against isotype-specific peptides. In addition to the previously observed α- and ε-subspecies, mesangial cells also express the δ- and ζ-isoenzymes of PKC. On exposure to phorbol 12,13-dibutyrate (PDB) a complete depletion of PKC-δ is observed within 8 h. Removal of PDB results in a recovery of PKC-δ. In contrast, PKC-ζ is unaffected by addition or removal of PDB.

Protein kinase C; Isoenzyme; Mesangial cell

1. INTRODUCTION

Molecular cloning analysis has shown that protein kinase C (PKC) is a family of at least eight isoenzymes, all having closely related structures but differing in their individual enzymological properties [1,2]. Different tissue and cellular distributions have been found for the PKC subtypes, suggesting specific roles for each isoenzyme in cell regulation [1,2]. Nevertheless it has proven to be difficult to ascribe specific cellular responses to the activation of individual PKC isoforms. In glomerular mesangial cells PKC fulfills two important functions: it contributes to hormone-induced prostaglandin formation, and it acts as a negative feedback regulator of the inositol lipid signalling cascade (for review see [3,4]). Recently we have shown that mesangial cells express two PKC isoenzymes, PKC-α and -ε. No PKC-β and -γ isoenzymes were detected. By comparing down-regulation kinetics of PKC-α and -ε isoforms after phorbol ester treatment with the down-regulation of the cells' functional responses, we have suggested that PKC-α may negatively regulate hormone-stimulated $InsP_3$ generation, whereas PKC-ε may transmit activation of prostaglandin synthesis [5]. In this report, we demonstrate that mesangial cells in addition to PKC-α and -ε also express PKC-δ and -ζ isoenzymes. Whereas the kinetics of down-regulation of PKC-δ is similar to those of PKC-α, PDB does not affect PKC-ζ at all.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Phorbol 12,13-dibutyrate (PDB) was obtained from Calbiochem, Lucerne, Switzerland; all cell culture nutrients were from Boehringer-Mannheim, Germany; all other chemicals used were from Merck, Darmstadt, Germany, or from Bio-Rad, Glattbrugg, Switzerland.

2.2. *Cell culture*

Rat renal mesangial cells were cultured as described previously [6]. In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were characterized [7] and used for further processing. The cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and bovine insulin (0.66 U/ml). For the experiments, passages 10 to 16 of mesangial cells were used.

2.3. *Peptide synthesis and generation of antibodies*

Synthetic peptides based on the C-terminal sequence deduced from brain cDNAs of rat PKC-δ (659-KGFSFVNPKYEQFLE-673) and rat PKC-ζ (577-GFEYINPLLLSAEESV-529) were synthesized on an ABI 431 peptide synthesizer, coupled to Keyhole-limpet haemocyanin by glutaraldehyde and used to immunize rabbits as described previously [8].

2.4. *Immunoblot analysis of PKC isoenzymes*

Confluent mesangial cells in 100 mm-diameter dishes were washed with phosphate-buffered saline (PBS) and incubated for 24 h in 10 ml DMEM containing 0.1 mg of fatty acid-free BSA/ml and the indicated concentrations of phorbol esters. After incubation, the cells were washed three times with PBS and incubated with fresh DMEM for the indicated recovery periods. Thereafter 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 benzamidine) with a rubber policeman. Fractionation into cytosolic and particulate fractions was performed as described previously [5]. Protein concentration was determined by the method of Bradford [9]. The PKC fractions were subjected to SDS-PAGE (8%

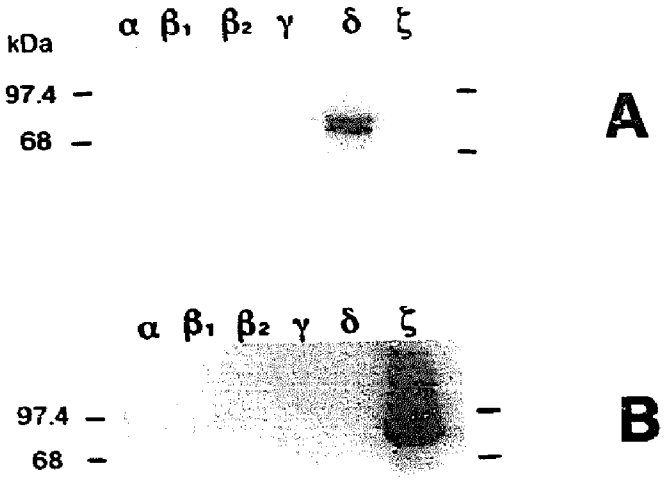

Fig. 1. Characterization of anti-PKC-δ and anti-PKC-ζ antisera specificity. Specificities of anti-PKC antisera towards their respective antigens were tested using insect cell extracts expressing recombinant PKC-α, PKC-β_1, PKC-β_2, PKC-γ, PKC-δ and PKC-ζ [28]. Identical amounts of protein (10 μg) were resolved on SDS-PAGE and immunoblotted using the anti-PKC-δ (A) or anti-PKC-ζ (B) antisera. Bands were detected with ^{125}I-labelled anti-rabbit antibodies.

acrylamide gel) as described by Laemmli [10], and blotted on to a nitrocellulose paper for 1 h at 250 mA using a Bio-Rad Transblot apparatus. Nitrocellulose filters were blocked with 3% (w/v) BSA in PBS for 1 h and incubated for 4 h with antiserum reactive with PKC-δ or -ζ, respectively, in PBS containing 0.1% BSA. After washing with PBS containing 0.1% Tween 20, the filters were incubated for 30 min with horseradish peroxidase-conjugated anti-rabbit IgG antibodies, and colour development was performed as described [5]. Alternatively, immunoreactivity was analyzed using ^{125}I-labelled anti-rabbit antibodies (Amersham).

3. RESULTS

The PKC isoenzymes in mesangial cells were characterized by immunoblotting analysis using polyclonal anti-peptide antibodies. For the δ- and ζ-isoenzymes, the antibodies were raised against synthetic peptides corresponding to specific sequences of the C-terminal part of rat brain PKC. Specificities of anti-PKC antisera towards their respective antigens as well as the other PKC subtypes were tested using insect cell extracts expressing recombinant PKC-α, PKC-β_1, PKC-β_2, PKC-γ, PKC-δ and PKC-ζ. Identical amounts of each individual PKC subtype were loaded onto SDS-PAGE and transferred to nitrocellulose. Each anti-peptide antibody reacted specifically with its own antigen, showing no cross-reactivity with the other PKC isoenzymes (Fig. 1). Preliminary data indicate that the antibodies also do not cross-react with PKC-ε or PKC-η (data not shown). Immunoblot analysis of cytosolic and particulate fractions of mesangial cells displayed strong immunoreactivity for both isotypes, 78 kDa PKC-δ and 68 kDa PKC-ζ (Fig. 2). Whereas PKC-δ immunoreactivity is predominantly present in the particulate fraction, PKC-ζ is preferentially located in the cytosolic fraction (Fig. 2, lane 1). A 24 h incubation with PDB resulted in an almost complete (>90%) down-regulation of PKC-δ (Fig. 2, lane 2). In contrast, PDB treatment for 24 h did not induce down-regulation of PKC-ζ. After 24 h PDB exposure cells were washed extensively, fresh medium was added and the cells were allowed to recover from the down-regulation regimen. PKC-δ reappeared in the particulate fraction within 4 h (Fig. 2, lane 4) and resumed control levels in the membrane compartment after 32–48 h (lanes 6 and 7). There was only a partial recovery of PKC-δ in the cytosolic fraction. Again, PKC-ζ was unaffected by the removal of PDB (Fig. 2). The data in Fig. 2 clearly demonstrates that a 24 h treatment of mesangial cells is sufficient to down-regulate PKC-δ. In order to get a more detailed time-course of PKC-δ depletion, additional time points of PDB exposure were examined. Fig. 3 shows that short-term exposure to PDB for up to 1 h results in an increase of membrane-bound PKC-δ, consistent with a translocation of the enzyme from the cytosolic to the particulate fraction. Long-term treatment with PDB results in a progressive loss of PKC-δ, with a complete depletion observed after 8 h.

4. DISCUSSION

Mesangial cells are a major determinant of the glomerular filtration rate. Morphologically, mesangial cells resemble vascular smooth muscle cells and are able to contract upon stimulation by vasoactive hormones [3]. When appropriate agonists, such as angiotensin II or vasopressin, bind to cell surface receptors like mesangial cells, they activate a phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate with the formation of $InsP_3$ and 1,2-diacylglycerol. The latter serves as endogenous activator of PKC [3,4]. Kuo et al. [11] were the first to report on the existence of PKC in the kidney. Like many other tissues, the kidney contains the α and β subspecies of PKC [12,13]. PKC was also shown to be present in the cytosolic fraction of cultured mesangial cells [14,15]. Recently we have shown that mesangial cells express PKC-α and PKC-ε isoenzymes. No PKC-β and PKC-γ isoforms have been detected [5]. PKC-α and PKC-ε displayed differential kinetics of down-regulation and recovery after long-term phorbol ester treatment in mesangial cells [5,16]. These kinetics correlated reasonably well with the time-courses of removal and recovery of the specific cellular functions ascribed to PKC activation, i.e. stimulation of prostaglandin synthesis and feedback inhibition of angiotensin II-stimulated $InsP_3$ formation. We therefore hypothesized that PKC-α may negatively regulate phosphoinositide hydrolysis, whereas PKC-ε may trigger prostaglandin generation [5,16]. In the present paper we report that mesangial cells express, in addition to the previously described PKC-α and PKC-ε, larger amounts of PKC-δ and PKC-ζ isotypes. The down-regulation ki-

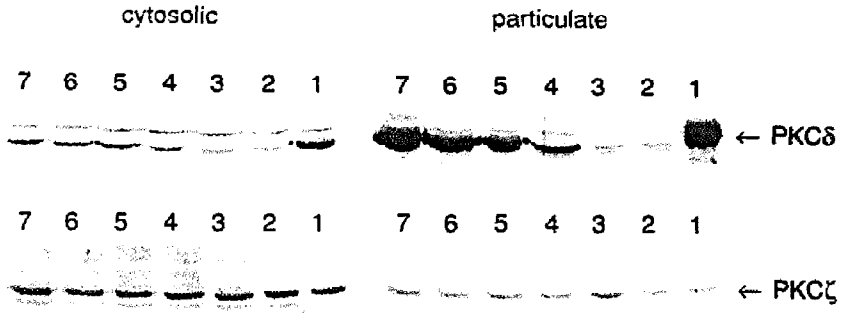

Fig. 2. Immunoblot detection of down-regulation and recovery of PKC-δ and -ζ in mesangial cells. Mesangial cells were treated with vehicle (lane 1) or PDB (500 nM) for 24 h (lanes 2–7) and after washing were incubated for recovery periods of 1 h (lane 3), 4 h (lane 4), 24 h (lane 5), 32 h (lane 6) or 48 h (lane 7), and the cytosolic and particulate fractions were prepared as described in section 2. Samples (70 μg of protein) were subjected to SDS-PAGE, transferred to nitrocellulose, and Western-blot analysis was performed using antiserum against PKC-δ and -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. It should be noted that the lower band in the upper panels represents PKC-δ.

netics of PKC-δ parallels closely those of PKC-α, thus suggesting that PKC-δ is an additional candidate for mediating feedback inhibition of $InsP_3$ production. Furthermore, we have shown that PKC-ζ is resistant to down-regulation by phorbol ester treatment. In this respect PKC-ζ behaves completely differently from all the other PKC isotypes in mesangial cells. Indeed preliminary structural and biochemical data indicate that PKC-ζ is related to, but distinct from, other isoforms of PKC. Ono et al. [17] reported that PKC-ζ expressed in COS-7 cells is unable to bind phorbol esters and displays a protein kinase activity that is independent of diacylglycerol. These data, together with our observations, indicate that results from long-term phorbol ester-treated cells, so called PKC down-regulated cells, must be interpreteted with caution. Cellular responses that are not altered subsequent to long-term phorbol ester treatment are not necessarily PKC-independent, but may be mediated by PKC isoenzymes that are resistant to down-regulation, e.g. PKC-ζ. PKC-ζ has been identified in nuclei of nerve cells [18], bovine neutrophils [19] and in human platelets [20], but no physiological function is known so far. The molecular weight of the PKC isoenzyme recognized by our PKC-ζ antiserum is approximately 68 kDa. This conforms well to the molecular size of 64 kDa reported for the PKC-ζ partially purified from COS cells [17].

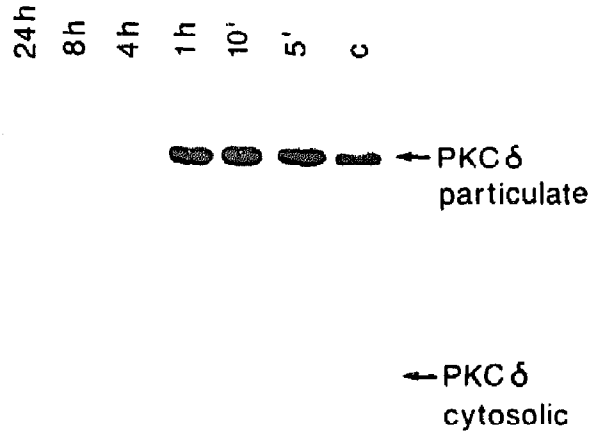

Fig. 3. Time-course of PKC-δ down-regulation by PMA in mesangial cells. Mesangial cells were treated with PMA (500 nM) for the indicated time periods, and the cytosolic and particulate fractions were prepared. Samples (70 μg of protein) were applied to SDS-PAGE and transferred to nitrocellulose. Immunoblots were developed by using antiserum against PKC-δ at a dilution of 1:1000. Bands were detected with ^{125}I-labelled anti-rabbit antibodies.

PKC-δ cDNA from rat brain was first cloned by Ono and colleagues [21] and also characterized by other groups [22,23]. Leibersperger et al. [24] reported on the purification of PKC-δ from the particulate fraction from porcine spleen. These latter authors also demonstrated the presence of PKC-δ in murine keratinocytes, bovine endothelial cells and several murine tissues, including the kidney [25]. As in mesangial cells, PKC-δ was located predominantly in the particulate fractions of the different tissues and cell lines investigated. The apparent weight of 78 kDa for PKC-δ in mesangial cells is consistent with the molecular size of PKC-δ purified from porcine spleen [24]. Very recently, PKC-δ was shown to be the major isotype of PKC expressed in hemopoietic cells [23]. Although PKC-δ appears to be a ubiquitously distributed PKC isotype [26], nothing is known about specific cellular roles of this isoenzyme. The main finding of the immunoblot analysis is that PKC-δ is down-regulated with kinetics resembling closely that of PKC-α [5], thus suggesting that PKC-α and PKC-δ are both candidates for triggering feedback inhibition of phosphoinositide hydrolysis. It has been reported that PKC-δ has a significantly lower sensitivity towards the PKC inhibitor, K252a, when compared to crude PKC preparations [27]. The development of more selective, isoenzyme-specific PKC inhibitors will help to identify the active PKC isotype unequivocally.

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REFERENCES

[1] Nishizuka, Y. (1988) Nature 334, 661–665.
[2] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C.J., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11.
[3] Pfeilschifter, J. (1989) Eur. J. Clin. Invest. 19, 347–361.
[4] Pfeilschifter, J. (1990) Klin. Wochenschr. 68, 1134–1137.
[5] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. J. 279, 441–445.
[6] Pfeilschifter, J., Kurtz, A. and Bauer, C. (1984) Biochem. J. 223, 855–859.
[7] Pfeilschifter, J. and Vosbeck, K. (1991) Biochem. Biophys. Res. Commun. 175, 372–379.
[8] Borner, C., Wyss, R., Regazzi, R., Eppenberger, U. and Fabbro, D. (1987) Int. J. Cancer 40, 344–348.
[9] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, L., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043.
[12] Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. 151, 973–981.
[13] Liqun, D., Stevens, J.L. and Jaken, S. (1991) Am. J. Physiol. 261, F679–F687.
[14] Orita, Y., Fujiwara, Y., Ochi, S., Tanaka, Y. and Kamada, T. (1985) FEBS Lett. 192, 155–158.
[15] Pfeilschifter, J. (1988) Biochim. Biophys. Acta 969, 263–270.
[16] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. Biophys. Res. Commun. 180, 1422–1428.
[17] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 3099–3103.
[18] Hagiwara, M., Ushida, C., Usuda, N., Nagata, T. and Hidaka, H. (1990) Biochem. Biophys. Res. Commun. 168, 161–168.
[19] Stasia, M.J., Strulovici, B., Daniel-Issakani, S., Peosin, J.M., Dianoux, A.C., Chambaz, E. and Vignais, P.V. (1990) FEBS Lett. 274, 61–64.
[20] Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erne, P. (1991) Biochem. Biophys. Res. Commun. 178, 878–883.
[21] Ono, Y., Fuji, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
[22] Olivier, A.R. and Parker, P.J. (1991) Eur. J. Biochem. 200, 805–810.
[23] Mishak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F. and Mushinski, J.F. (1991) Biochemistry 30, 7925–7931.
[24] Leibersperger, H., Gschwendt, M. and Marks, F. (1990) J. Biol. Chem. 265, 16108–16115.
[25] Leibersperger, H., Gschwendt, M., Gernold, M. and Marks, F. (1991) J. Biol. Chem. 266, 14778–14784.
[26] Mizuno, K., Kubo, K., Saido, T.C., Akita, Y., Osada, S., Kuroki, T., Ohno, S. and Suzuki, K. (1991) Eur. J. Biochem. 202, 931–940.
[27] Gschwendt, M., Leibersperger, H. and Marks, F. (1989) Biochem. Biophys. Res. Commun. 164, 974–982.
[28] Patel, G. and Stabel, S. (1989) Cell. Signalling 1, 227–240.