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Protein kinase C subtypes in endothelial cells

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Activation of protein kinase C (PKC) has been linked to the regulation of class II expression on endothelial cells by interferon-γ (IFN-γ). PKC subtypes in endothelial cells were analyzed using three different approaches, the immunoperoxidase staining of native and IFN-γ stimulated cells cultured on chamber slides as well as immuno- and Northern blotting. All approaches revealed that of the conventional subtypes, α is the predominant form of PKC in endothelial cells. Even though IFN-γ is able to induce PKC translocation to particulate fractions, no translocation was detected in histological stainings. Western blot studies as well as mRNA studies revealed that IFN-γ is unable to increase the total amount of PKC in endothelial cells.

Endothelial cell; Protein kinase C; Interferon-gamma; Diacylglycerol

1. INTRODUCTION

In signalling via phosphatidylinositol (PI)-dependent pathway, binding of ligand to its receptor results in the breakdown of phosphatidyl 4,5-bisphosphate (PIP_2) and formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The former elevates the intracellular Ca^{2+} concentration and the latter activates protein kinase C (PKC). Although the hydrolysis of phosphoinositolbisphosphate (PIP_2) was thought to be the sole mechanism for PKC activation, recent studies suggest that there are several additional signal pathways to provide DAG. For instance, phosphatidylcholine (PC) may also be hydrolysed to produce DAG [1]. Hydrolysis of PC by either phospholipases C or D can lead to DAG production [2–5]. Phospholipase C (PLC) hydrolysis of PC is followed by an increase in DAG and phosphorylcholine whereas increases in choline, phosphatidic acid (PA) and finally DAG suggest that phospholipase D is involved.

Protein kinase C is a family of closely related proteins that differ in tissue distribution, co-factor dependency and substrate specificity [6,7]. The relative distribution of PKC subspecies varies markedly with different areas of tissues and cell types examined. PKC subtypes α, βI, βII and γ were described first [8,9]. More recently also cDNA clones for δ, ε and ξ subtypes have been prepared [10]. The latest member of this family, PKCη, was described this year [11]. The conventional PKC subtypes α, β and γ are characterized by their requirement for Ca^{2+}, phospholipids and DAG. The other group of PKC isotypes δ, ε, ξ and η, the novel isotypes, seems to be independent of Ca^{2+} [11].

Earlier studies in this laboratory have revealed that protein kinase C is induced following IFN-γ treatment in endothelial cells [12]. Protein kinase C subtypes in non-stimulated and IFN-γ-stimulated endothelial cells are now described.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Four to 12-day-old DA rats were operated with a modification of the method of Kasten [13]. In some experiments the cells growing in flasks or Lab-Tek chamber slides (Miles Scientific, IL, USA) were stimulated with 100 U/ml recombinant rat IFN-γ (a gift from Dr P.H. v.d. Meide, Rijswijk, The Netherlands) for different time periods.

2.2. *Immunoperoxidase stainings*

Cells cultured in chamber slides were fixed with acetone for 10 min. All subsequent procedures were carried out inside a humidified box. The specimens were exposed for 30 min to mouse monoclonal antibodies: monoclonal anti-protein kinase C type α (type 3), type β (type 2) and type γ (type 1, all purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan). The secondary antibody used was peroxidase-conjugated rabbit anti-mouse Ig and the third antibody was peroxidase-conjugated goat anti-rabbit Ig. Aminoethyl carbatsole and hydrogen peroxide were used as a chromogen and substrate, respectively, to demonstrate antigen in cells. Mayer's Hemalaun (Merck, Darmstadt, Germany) was used to counter stain.

2.3. *Immunoblotting*

10×10^6 cells were lysed with 10 vols of SDS-PAGE sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.01% Bromophenol blue), mixed vigorously and boiled for 5 min. To shear the chromosomal DNA the sample was sonicated for 20 s at full force. The sample was then centrifuged at $10\,000 \times g$ for 10 min, the supernatant was recovered and frozen at −20°C. SDS-PAGE was made using an 8% gel and Mighty Small II slab gel electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, USA). Samples were applied to four wells. Rainbow protein molecular weight markers (92.5 kDa, 69 kDa, 46 kDa, 30 kDa, 21 kDa and 14.3 kDa) were used as standard.

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Electrophoretic transfer was made by Mini Transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) following the manufacturer's instructions using nitrocellulose filters (Hybond) and transfer buffer containing Tris 25 mM, glycine 190 mM, methanol 20% (pH 8.3). After blotting the filter was allowed to dry completely in order to minimize the chance of the transferred proteins being lost during subsequent steps. When the filter was cut in slices, rinsed several times with PBS and incubated for 2 h in the blocking solution (3% BSA/PBS) with agitation, washed twice with PBS and incubated with different anti-PKC antibodies overnight. After the PKC subtype antibody incubation filter pieces were washed twice with PBS, exposed to peroxidase-conjugated rabbit anti-mouse antibody for 1 h, washed 4 times with PBS and exposed to peroxidase-conjugated goat anti-rabbit antibody for 1 h. After the final incubation, filter pieces were washed once more and developed with aminoethyl carbatsole and hydrogen peroxide.

2.4. *Extraction and analysis of RNA*

Poly (A^+) RNA was extracted from 10^7–10^8 cells. Where indicated IFN-γ was added at 100 U/ml, H-7 at 2 or 20 μM and cycloheximide at 150 μg/ml. Poly (A^+) mRNA was bound to oligo-dT cellulose directly from cell lysates and eluted as described [14]. 20 μg of mRNA was dissolved in sample buffer (20 mM morpholinopropanesulfonic acid, 50% formamide, 2.2 M formaldehyde, 5% Ficoll, 5 mM EDTA, 0.04% Bromophenol blue), size-fractionated in formaldehyde-agarose (0.8%) gels and transferred to Immobilon-N transfer membranes (Millipore Corporation, Bedford, MA) in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 24 h. Hybridizations were carried out at +42°C for 20 h in a hybridization mixture containing 5 × Denhardt's solution (100 × Denhardt's solution is 2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 5 × SSC, 100 mM PO_4 (21.9 mM K_2HPO_4 and 78.1 mM KH_2PO_4), 50% formamide, 100 μg/ml salmon sperm DNA and 100 μg/ml yeast transfer RNA. Post-hybrization washes were done twice at room temperature, twice at +60°C with 2 × SSC containing 0.1% SDS and twice with 0.2 × SSC containing 0.1% SDS at +60°C for 15 min. The filters were exposed to Kodak XAR-5 film at −70°C for periods of 24 h to 14 days. Radioactive signals were quantitated from the autoradiograms with

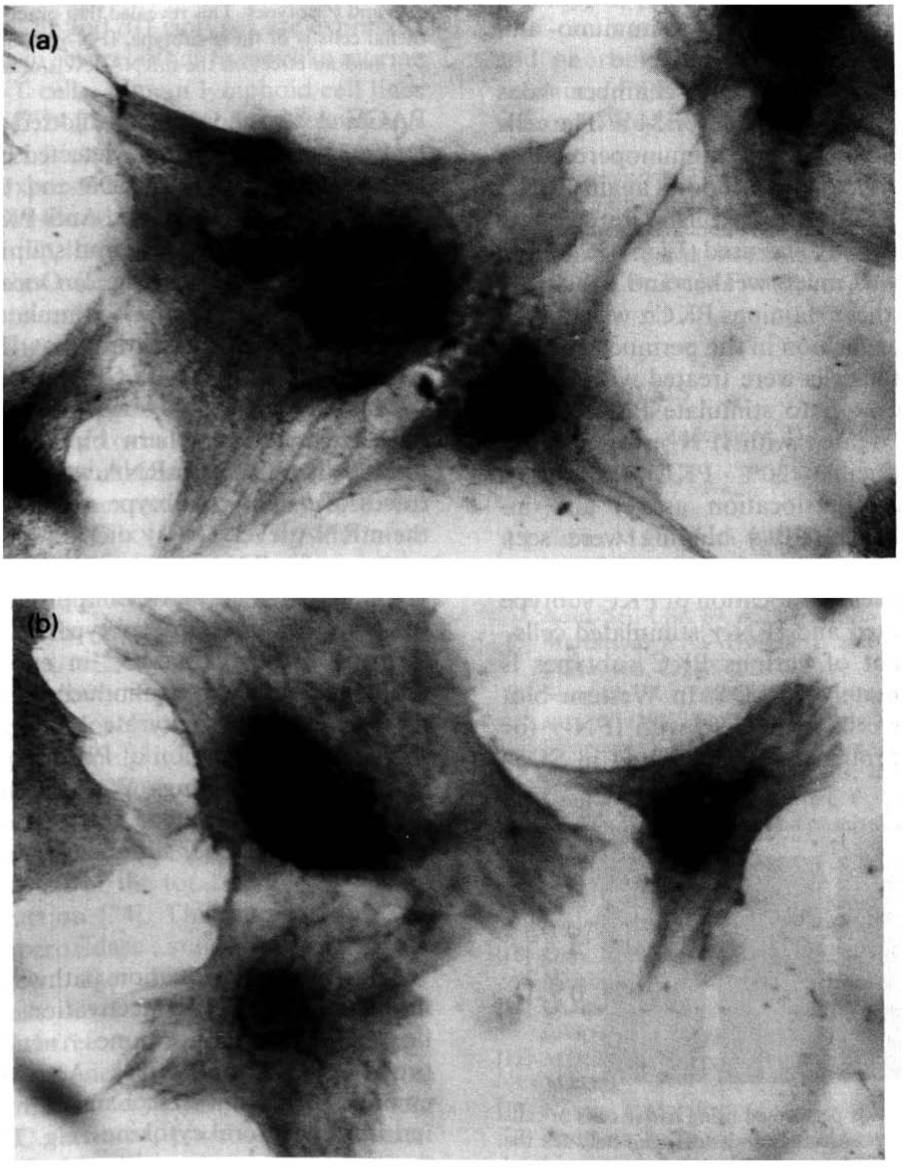

Fig. 1. Endothelial cells on chamber slides were stained with antibodies to conventional PKC isotypes. Anti-α antibody staining was strongly positive when both control cells (Fig. 1a) or IFN-γ-treated cells (Fig. 1b) were used, PKCα being mainly associated to the perinuclear area. Stainings with anti-β and anti-γ antibodies were weak and negative, respectively (data not shown). Negative control with an irrelevant antibody was also done and was found to be blank.

a densitometric scanner (Helena Laboratories, Beaumont, TX). The PKC α, β and γ probes were purchased from American Type Culture Collection. The insert sizes in pUC vector were 1.29, 1.70 and 1.40 kb, respectively. The cDNA for glyceraldehyde-3-phosphate-dehydrogenase (GADPH [15]), which has been shown to be fairly invariably expressed and stimulated only by insulin in adipocyte cultures (ref) was a gift from Dr P. Fort (Montpellier, France) and used as an internal control for RNA loading. Nick-translations of cDNA using [α-^{32}P]dCTP (3000 Ci/mmol) were carried out according to the manufacturer's instructions (Amersham, UK).

3. RESULTS

Activation of protein kinase C has been linked to regulation of class II expression on endothelial cells by IFN-γ. I wanted to analyse further, which PKC subtypes are involved in endothelial activation. To do this three different approaches were used, the immunoperoxidase staining of native and IFN-γ-stimulated cells cultured on chamber slides as well as immuno- and Northern blottings.

Endothelial cells were transferred to chamber slides and cultured in the presence of serum for 24 h. The cells were then fixed and stained with an immunoperoxidase technique applying monoclonal antibodies against PKC subtypes α, β and γ. Endothelial cells were strongly stained when anti-α antibody was used (Fig. 1). Staining with anti-β antibody was much weaker and subtype γ was not detectable. In these stainings PKCα was mainly associated in a granular fashion in the perinuclear area. In some experiments the cells were treated with IFN-γ for 5 min, 15 min or 24 h to stimulate PKC. When endothelial cells were treated with IFN-γ for 5 min a constant and reproducible 30% PKC translation (measured by histone translocation assay) and increased phorboldibutyrate (PBt_2) binding were seen [16]. In the same kind of experiment here, no difference was recorded in the subcellular location of PKC subtype α between non-stimulated and IFN-γ stimulated cells.

The molecular weight of various PKC subtypes is known to be approximately 76 kDa. In Western blot analysis native cells or cells stimulated with IFN-γ for 24 h were lysed, the proteins were separated in SDS-PAGE electrophoresis and blotted to nitrocellulose filters. A positive band was detected between the molecular weight markers 92 kDa and 69 kDa when anti-PKCα antibody was used. Anti-PKCβ antibody staining was barely detectable and staining with anti-PKCγ antibody was negative (Fig. 2). Once again no difference was seen between IFN-γ stimulated and native cells (data not shown) indicating that IFN-γ could not increase the amount of PKC in endothelial cells.

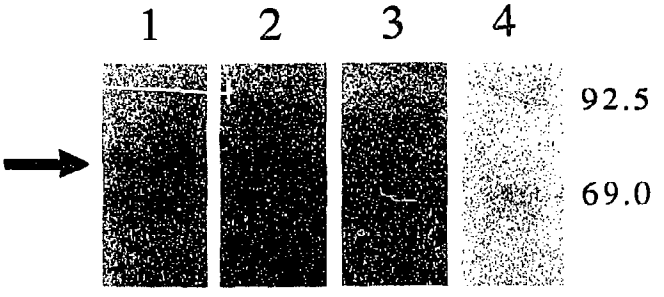

Fig. 2. Western blot analysis was performed using native cells or cells stimulated with IFN-γ for ā positive band was detected between the MW markers 92 kDa and 69 kDa when anti PKC-α antibody was used (lane 1). Anti-PKC-β staining was barely detectable (lane 2) and anti-PKC-γ-staining was negative (lane 3). Lane 4 represents non-specific background. 24 h IFN-γ stimulus was not able to increase the amount of PKC in endothelial cells (data not shown).

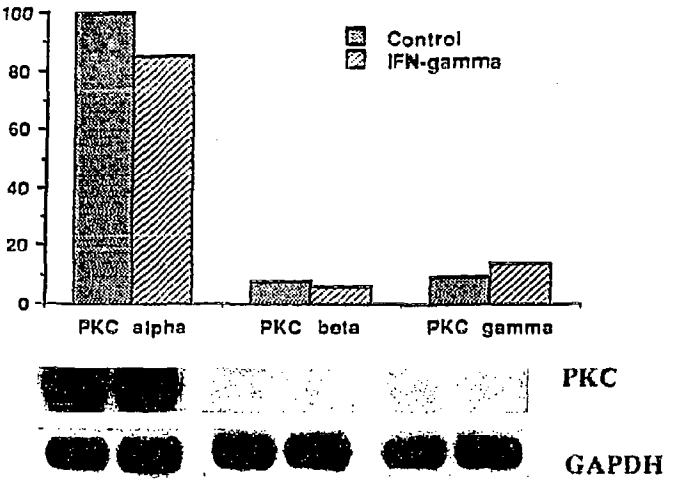

Fig. 3. Northern blot analysis was done using cDNA probes for PKC α, β and γ isotypes. This revealed that practically all mRNA in endothelial cells is of the α-subtype. IFN-γ induction of endothelial cells had no effect on the mRNA levels of α, β and γ subtypes.

Finally poly(A$^+$)RNA was isolated from cultured endothelial cells. Northern blot analysis revealed that practically all PKC-mRNA was of the α subtype. Only traces of β- and γ-subtype message were detectable at the mRNA level. IFN-γ did not have any effect on the amount of mRNA message of the subtypes (Fig. 3).

Taken together, all three approaches revealed that of the conventional PKC subtypes, α is the predominant form of protein kinase C in endothelial cells. Even though IFN-γ is able to induce PKC translocation and increase phorboldibutyrate binding, I was unable to show any translocation of PKC to particulate fractions in histological stainings. Western blot studies as well as mRNA studies revealed that IFN-γ is unable to increase the total PKC amount in endothelial cells.

4. DISCUSSION

The signal transduction pathway involving calcium mobilization and PKC activation is a mechanism utilized by several hormones, neurotransmitters, and growth factors [2,17–19]. Activation of PKC has emerged as a major mechanism in transducing signals induced by several cytokines, e.g. TNF, IL-1 and interferons [2,17].

A few interferon-induced transmembrane signalling processes are known to occur via the PKC pathway. In human fibroblasts, α and β interferons stimulate a tran-

sient two- to threefold increase in the concentration of DAG and IP_3 [19]. PKC is also known to be involved in murine macrophage activation by IFN-β [20]. Furthermore, IFN-α selectively activates the β isoform of PKC through phosphotidylcholine hydrolysis in HeLa cells [4]. IFN-γ induces DAG release and IP_3 formation within 30 s in Daudi cells and it also modulates PKC activity in murine macrophages [19,21]. We have reported that PKC mediates IFN-γ induced class II expression in endothelial cells [12,16]. In other cell types than endothelial cells the role of PKC activation to MHC class II upregulation is controversial [22].

The relative activity and individual pattern of expression of multiple PKC subspecies in several tissues and cell types have recently been examined extensively. PKC α is widely distributed in many tissues and cell types like in brain tissue, HL-60 cells, T cells and platelets [7,23–25]. In contrast, PKC βI and βII isotypes are expressed in the brain as well as in several other tissues like murine thymocytes, human T cells, human lymphoid cell lines and human platelets [7,24,26,27]. PKC β is the predominant isotype in spleen tissue and in leukocytes. PKC with γ sequence is expressed solely in the central nervous tissue, and is not found in any other tissue or cell type analysed so far [7]. In general, it seems to be that one cell type contains more than one PKC isotype. These subspecies apparently show a distinct intracellular location, depending on the state of differentation or proliferation. Very little is known about the distribution of the novel isoenzymes encoded by δ, ε, ξ and η sequences. PKC ε is known to be expressed in murine thymocytes as well as in cultured fetal chick neurons [28,29]. Mouse cell lines of neuronal origin express PKC ε and ξ in addition to PKC α [30]. PKC η, the latest member of the family is predominantly expressed in lung and skin [11]. The results presented in this paper suggest that PKC-α is the predominant isotype in endothelial cells.

Protein kinase C activity has been linked to its translocation from cell cytosol to particulate fractions of the cell, to cell membrane [31,32] or to nuclear envelope [33]. However, the majority of PKC activity in non- dividing cultured porcine aortic endothelial cells is found in the cytoplasm, whereas in vigorously proliferating cells, more than half of the total activity is found in the membrane fraction [34]. This agrees with our results in immunoperoxidase stainings where no translocation was seen after IFN-γ treatment. We plated endothelial cells on chamber slides sparsely to detect single cells and therefore these cells were dividing. Furthermore only a 30% increase in translocation was seen in bovine pulmonary artery endothelial cells when treated with the PKC activator, phorbol 12-myristate 13-acetate [35], measured by histone translocation and also in our endothelial cells when treated with IFN-γ [16]. In this system PKC activation reduces already after 10 min after IFN-γ stimulation. PKC activation in this endothelial cell system is so transient that it is unlikely that new PKC protein synthesis would have had time to take place. Stimulation of peritoneal murine macrophages with IFN-γ leads to PKC activation but did not alter the subcellular distribution of the enzyme [21]; in rat brain and lymphocytes half of the tissue PKC contents is continuously bound to membranes [36]. It is also known that some biological responses mediated by PKC may be achieved by activation of less than 5% of the total enzyme activity [37]. On the contrary in human T cells about a 10-fold translocation has been observed in histone translocation assay [31] and in these cells this subcellular redistribution is also seen in immunocytochemical stainings [32]. The results presented in this paper suggest that PKCα is the predominant subtype in endothelial cells, and that there is some PKCβ but no γ subtype present. Even though IFN-γ is able to cause PKC activation (measured by histone translocation- and phorbol binding assays) it does not increase the amount of PKC as measured by immunoperoxidase staining, Western- or Northern blotting.

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