Heparin down-regulates the phorbol ester-induced protein kinase C gene expression in human endothelial cells: enzyme-mediated autoregulation of protein kinase C-α and -δ genes[1]

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Abstract Overexpression of protein kinase C-α and protein kinase C-δ has been shown to modulate a number of biological effects, including the cell growth and differentiation. We hypothesized that heparin, a potent antimitogenic drug, could affect the cell proliferation by inhibiting the expression of specific protein kinase C genes. Heparin, markedly but not completely, inhibited the serum-stimulated protein kinase C-α and -δ mRNA expression. Protein kinase C inhibition or down-regulation significantly decreased the serum-induced protein kinase C isoenzyme gene expression. Heparin failed to inhibit the residual effect of serum that was resistant to the above-mentioned treatments. Phorbol 12-myristate 13-acetate elicited an increase of protein kinase C isoenzyme gene expression that was completely prevented by protein kinase C inhibition or down-regulation. Heparin dose-dependently counteracted and ultimately abolished the increase in the protein kinase C isoenzyme gene expression elicited by phorbol 12-myristate 13-acetate. These results suggest that the inhibition of an autoregulatory role wielded by protein kinase C on the protein kinase C-α and -δ gene expression might represent a possible mechanism by which glycosaminoglycans modulate the cell growth.

Key words: Heparin; Protein kinase C gene expression; Endothelial cell

1. Introduction

Protein kinase C (PKC) is a multigene family of serine/threonine kinases which plays a fundamental role in a variety of cellular functions [1]. PKC isoenzymes are differentially expressed and respond differently to physiological stimuli in various tissues and cell types [2], supporting the view that different PKC isoforms subserve distinct functional roles within cells. It is now recognized that at least 10 different isoforms that have been identified by molecular cloning may contribute to the PKC activity [3–5]. Conventional PKCs (α, βI, βII and γ) are Ca^{2+}-dependent phospholipid-activated enzymes [1,6]. Novel PKCs (δ, ε, η and θ), lacking the C2 Ca^{2+} binding domain are Ca^{2+}-independent diacylglycerol (DAG)-activated isoenzymes. Atypical PKCs (ζ and λ/ι), which are both Ca^{2+}- and DAG-independent enzymes, have been found to be activated by phosphatidylserine and other lipid-derived mediators [1,6,7]. In different cell types, including human endothelial cells (HEC), vascular smooth muscle cells (VSMC) and fibroblasts, PKC plays a crucial role in the modulation of cell growth and differentiation, being implicated both in proliferative and anti-proliferative responses [8,9]. In addition, PKC activation by phorbol esters induces migration, proliferation [10] and tube formation in cultured endothelial cells [11], as well as angiogenesis in vitro [12,13]. The crucial role of PKC in transducing growth regulatory signals is also highlighted by the finding that the down-regulation of PKC-dependent pathways elicits growth inhibitory responses. In particular, glycosaminoglycan-mediated inhibition of cell proliferation [14–16] and angiogenesis [17] has been associated, at least in part, to the ability of heparin and heparan sulfate to counteract PKC-mediated events [18–20]. In this regard, we have recently shown that heparin markedly reduced both the serum- and phorbol ester-stimulated DNA synthesis in HEC by eliciting the down-regulation of the PKC-mediated ornithine decarboxylase gene expression (ODC) [21]. The elucidation of the effects induced by growth regulators on the expression of selected PKC genes is now becoming an area of inquiry. In the present study, the possibility that PKC genes may be targeted for the action of heparin was investigated in cultured HEC. Here, we demonstrate that heparin was able to inhibit both the serum- and phorbol 12-myristate 13-acetate (PMA)-stimulated isoenzyme gene expression and hypothesize that the polyelectrolyte may affect the PKC-mediated autoregulation of PKC genes.

2. Materials and methods

2.1. Cell culture and exposure

HEC were isolated from human umbilical cords and cultured as previously described [22]. Briefly, HEC were detached from the interior of the umbilical vein of a 30 cm segment cord by treatment for 10 min at 37°C with 0.05% (w/v) collagenase type II from *Clostridium hystolyticum* (Gibco BRL, Paisley, UK) in medium M199 (Gibco BRL, Paisley, UK) containing 100 U/ml of penicillin G sodium salt and 100 μg/ml streptomycin sulfate (Sigma Chemical, St. Louis, MO, USA). HEC were harvested at 1000×*g* for 10 min and finally resuspended in 5 ml medium M199 supplemented with 10% (v/v) foetal calf serum (FCS), 10% (v/v) newborn-calf serum (Gibco BRL, Paisley, UK), 2 mM glutamine and antibiotics. Cells were then plated in 25 cm^2 tissue culture flasks (Falcon, Oxnard, CA, USA) and cultured in an atmosphere of 5% CO_2/95% air. When confluent, HEC were subcultured at a split ratio of 1:2 by a brief treatment with 0.1% trypsin

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Table 1

Gene	Deoxyoligonucleotide sequences	Product base pair	Amplification condition
PKC-α	Forward, 5′-CGACTCTCTGTAGAAATCTGG-3′ Reverse, 5′-CACCATGGTGCACTCCACGTC-3′	443	95°C for 30 s 58°C for 30 s 72°C for 1 min
PKC-δ	Forward, 5′-AAAGGCAGCTTCGGGAAGGT-3′ Reverse, 3′-TGGATGTGGTACATCAGGTC	260	95°C for 30 s 58°C for 30 s 72°C for 1 min
GAPDH	Forward, 5′-CCACCCATGGCAAATTCCATGGCA-3′ Reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′	598	95°C for 30 s 58°C for 30 s 72°C for 1 min

plus 0.02% EDTA in phosphate buffered saline (PBS) (120 mM NaCl, 2.5 mM KCl, 8.5 mM NaH_2PO_4, 1.5 mM KH_2PO_4), pH 7.3. Cultured cells were identified as endothelial by their typical cobblestone appearance and production of Von Willebrand factor as measured by an enzyme-linked immunosorbent assay [23]. Subconfluent cells were synchronized following a 48 h incubation in serum-free medium M199 containing 0.2% (w/v) bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO, USA). Synchronized subconfluent HEC grown in 25 cm^2 culture flasks were stimulated to growth for the indicated time intervals in the presence of medium M199 containing 10% FCS or in a serum-free medium containing 100 nM PMA (Sigma Chemical, St. Louis, MO, USA). Under the same experimental conditions, synchronized subconfluent HEC also received different concentrations of heparin as described in the figure legend. PKC down-regulation was accomplished by exposing HEC to serum-free medium containing 1 μM PMA during the last 24 h of cell synchronization [24]. The PKC inhibitors chelerythrine or calphostin C (Alexis corporation, San Diego, CA, USA) were added at a concentration of 2.5 μM and 1 μM, respectively, during the last 4 h of cell synchronization.

2.2. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) assessment of mRNAs

Total RNA was extracted at the indicated time points, according to the procedure described by Chomczynski and Sacchi [25]. The PKC mRNA expression was evaluated by RT-PCR [26]. Total RNA (1 μg) from synchronized subconfluent HEC treated as indicated, was reverse-transcribed for 45 min at 37°C. The reaction was performed in a solution of 25 μl, containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.2 mM of each dNTP, 0.1 μg of oligo dT, 200 U of M-MLV reverse transcriptase (Gibco BRL, Paisley, UK). The reaction mixture was then heated at 95°C for 5 min to inactivate the enzyme. PCR amplification was performed in 25 μl of a reaction mixture containing 5 μl of the reverse transcribed cDNA, 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Gibco BRL, Paisley, UK), 0.2 mM of each dNTP and 50 pmol of each sense and antisense primers that were previously dissolved in TE solutions (Tris 10 mM pH 8.0, EDTA, 1 mM pH 8.0). The number of amplification cycles was determined experimentally for each primer pair by establishing the point at which exponential accumulation plateaus. Aliquots of the PCR reactions, including 0.5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) were taken after 10, 15, 20, 25, 30, 35 and 40 cycles. Then, [α-^{32}P]dCTP-labelled PCR products were electrophoresed on 2% agarose gels. Ethidium bromide-stained bands were excized under UV light and the radioactivity incorporated into PCR products was determined by β-scintillation counting. Under these experimental conditions, and using 30 PCR cycles, [α-^{32}P]dCTP-labelled time course experiments revealed that the products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PKC-α and -δ amplification were all within the linear phase of the reaction. Indeed, similar conditions are previously reported for a semiquantitative analysis of gene expression [27,28]. The position of PCR fragments was evaluated by comparison with a DNA molecular weight marker (Gibco BRL, Paisley, UK). GAPDH mRNA was used for each sample as an internal control for the mRNA integrity and equal loading. The levels of radioactivity incorporated into PKC-δ and -α products were normalized by comparison with the levels of radioactivity incorporated into the GAPDH product from the same sample. Specific primers directed against human sequences for PKC-α, -δ and GAPDH were previously described [29]. Their sequences, PCR reaction conditions and the size of generated fragments are shown in Table 1.

2.3. Statistical analysis

The statistical analysis of the data was performed by using the unpaired Student's t test, assuming a P value less than 0.05 as the limit of significance. Data are expressed as mean ± S.E.

3. Results

To assess that PCR reactions for GAPDH, PKC-α and -δ were in the linear phase, 1 μg RNA sample from synchronized subconfluent HEC, stimulated with serum for 1, 2, 4, 6, 12 and 24 h was reverse-transcribed. 5 μl of the resulting cDNA was amplified for 10, 20, 25, 30, 35 and 40 cycles. Fig. 1 reports the expression of the PKC-δ and -α genes following a serum treatment of 1 or 6 h, respectively, corresponding to

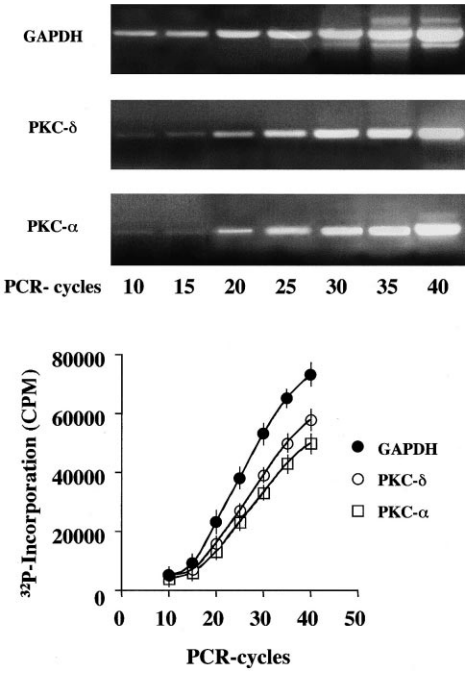

Fig. 1. Upper, representative ethidium bromide-stained gel of the reaction products obtained using 5 μl of the reverse transcriptase (RT) products after the indicated cycles of amplification for GAPDH, PKC-α and PKC-δ. Lower, graph demonstrating the linearity of reaction products obtained using 5 μl of RT product after the indicated cycles of [α-^{32}P]dCTP-labelled PCR amplification for GAPDH, PKC-α and PKC. Synchronized subconfluent HEC were stimulated with 10% FCS for 1, 2, 4, 6, 12 and 24 h. Then, the total RNA was extracted and processed for RT-PCR analysis. ● GAPDH, HEC were stimulated with 10% FCS for 6 h. PKC-δ, HEC were stimulated with 10% FCS for 1 h. PKC-α, HEC were stimulated with 10% FCS for 6 h. Data are the mean ± S.E. of four different experiments. CPM, counts per minute.

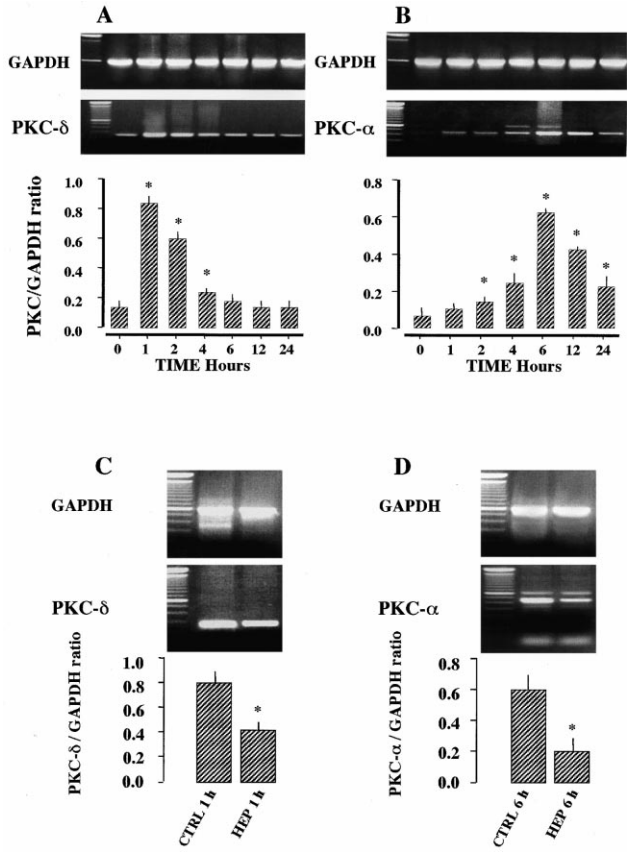

Fig. 2. A, B: time course of the effect of 10% FCS on PKC-δ (A) and PKC-α (B) mRNA expression in HEC. At the indicated time points, total RNA was extracted and processed for RT-PCR analysis. The upper part of each panel shows ethidium bromide-stained gels of the reaction products obtained using 5 μl of the RT products after 30 cycles of PCR amplification for GAPDH, PKC-δ (A) and PKC-α (B). The lower parts of A and B show serum-induced expression of PKC-δ (A) or PKC-α (B) mRNA detected by [α-^{32}P]dCTP-PCR (30 cycles) using 5 μl of RT product. Data represent the mean ± S.E. of four different experiments. *, significantly different from time 0. C, D: The effect of heparin (HEP) on the serum-mediated PKC-δ (C) and -α (D) mRNA expression in HEC. Cells were stimulated with 10% FCS for 1 h (C) or 6 h (D), in the absence (CTRL) or presence of 200 μg/ml heparin. The upper part of each panel reports ethidium bromide-stained gels of the reaction products obtained using 5 μl of the RT products after 30 cycles of PCR amplification for GAPDH, PKC-δ (C) and PKC-α (D). The lower parts of C and D show the expression of PKC-δ (C) or PKC-α (D) mRNA detected by [α-^{32}P]dCTP-PCR (30 cycles) using 5 μl of RT product. Data represent the mean ± S.E. of six different experiments. *, significantly different from CTRL. In A–D, individual results were normalized to GAPDH mRNA detected in each sample and expressed as a ratio to GAPDH.

the maximal effect of serum on the expression of each PKC gene. After the demonstration of linear PCR conditions at 30 cycles (Fig. 1), we investigated the expression of PKC-α and PKC-δ mRNA in synchronized subconfluent HEC that were cultured with 10% FCS, in the absence or presence of heparin. Serum elicited a time-dependent increase in PKC-α and -δ mRNAs (Fig. 2A, B). In the presence of serum, the PKC-δ gene expression reached a maximum at 1 h, thereafter progressively declined and returned to the control value at 12 h (Fig. 2A). Serum-induced overexpression of PKC-α mRNA was already evident at 2 h, but peaked after 6 h of treatment (Fig. 2B). The addition of heparin (200 μg/ml) to the incubation medium significantly, although not completely, inhibited the serum-stimulated PKC-δ and -α gene expression (Fig. 2C, D). We have previously shown that heparin failed to abolish completely the serum-stimulated DNA synthesis in HEC and

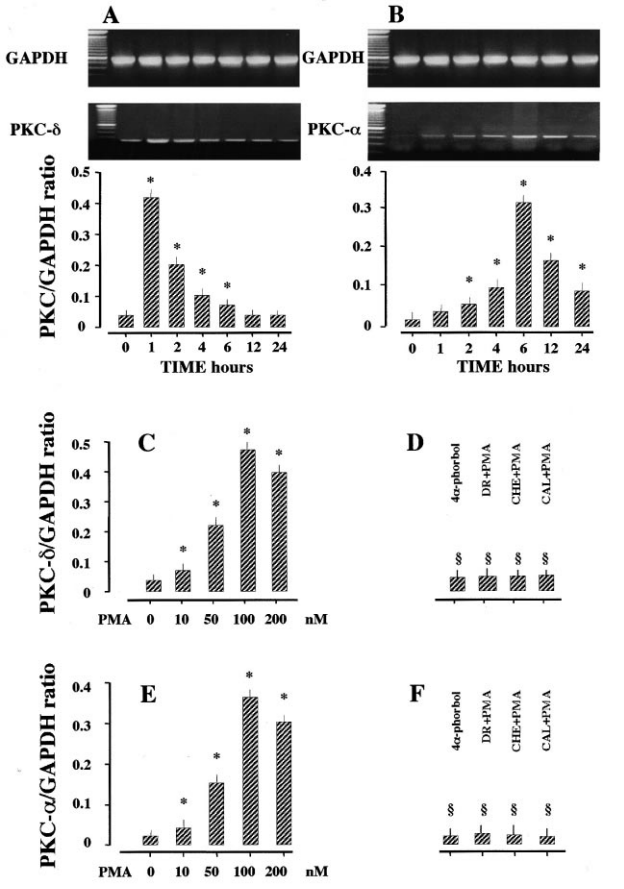

Fig. 3. A, B: time course of the effect of 100 nM PMA on PKC-δ (A) and -α (B) mRNA expression in HEC. At the indicated time points, total RNA was extracted and processed for RT-PCR analysis. The upper part of each panel shows ethidium bromide-stained gels of the reaction products obtained using 5 μl of the RT products after 30 cycles of PCR amplification for GAPDH, PKC-δ (A) and PKC-α (B). The lower parts of A and B report the expression of PKC-δ (A) and PKC-α (B) mRNA, detected by [α-^{32}P]dCTP-PCR (30 cycles) using 5 μl of RT product. Data represent the mean ± S.E. of four different experiments. *, significantly different from time 0. C, E: dose-response of the PMA effect on the PKC-δ (C) and -α (E) mRNA expression in HEC. Cells, cultured in a serum-free medium, were stimulated with the indicated concentrations of PMA for 1 h (C) or 6 h (E). Total RNA was extracted and processed for RT-PCR analysis. Expression of PKC-δ (C) and -α (D) mRNA was detected by [α-^{32}P]dCTP-PCR (30 cycles), using 5 μl of RT product. Data represent the mean ± S.E. of four different experiments. *, significantly different from 0 nM PMA. D, F: inhibition of the PMA-induced PKC-δ (D) or -α (F) mRNA expression by chelerythrine (CHE) (2.5 μM), calphostin C (CAL) (1 μM) or PKC down-regulation (DR). 4α-phorbol, HEC were stimulated for 1 h (D) or 6 h (F) with 100 nM of the inactive phorbol ester; DR+PMA, down-regulated HEC were stimulated for 1 h (D) or 6 h (F) with 100 nM PMA; CHE+PMA, chelerythrine-treated cells were stimulated for 1 h (D) or 6 h (F) with 100 nM PMA; CAL+PMA, calphostin C-treated cells were stimulated for 1 h (D) or 6 h (F) with 100 nM PMA. Data represent the mean ± S.E. of four different experiments. §, significantly different from 100 nM PMA. In A–E, results were normalized to the GAPDH mRNA expression detected in each sample and expressed as a ratio to GAPDH.

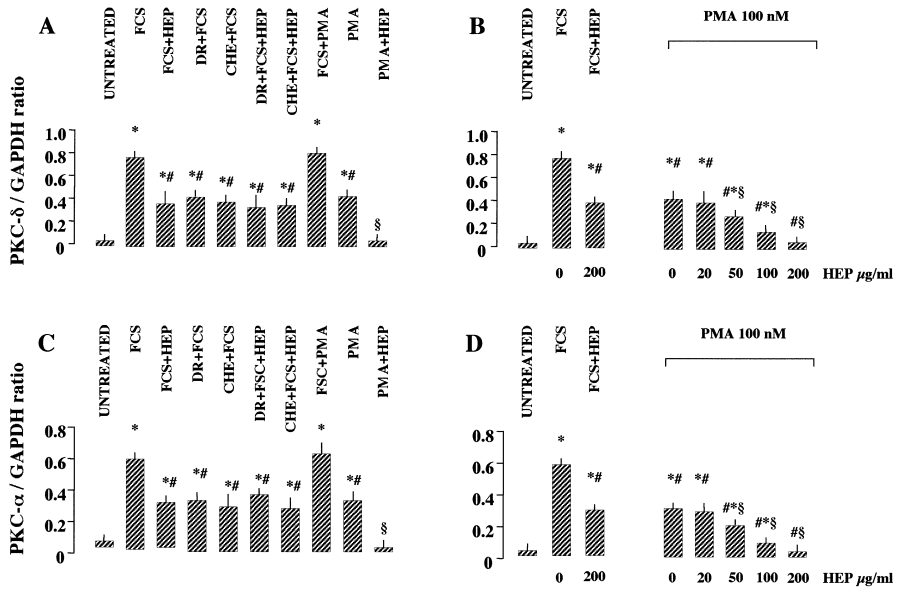

Fig. 4. A, C: inhibition of FCS-stimulated PKC-δ (A) and -α (C) mRNA expression by chelerythrine (CHE) (2.5 μM) or PKC down-regulation (DR) in HEC. Expression of PKC-δ (A) and -α (C) mRNA was detected by [α-^{32}P]dCTP-PCR (30 cycles) using 5 μl of RT product. UNTREATED, untreated cells; FCS, HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS; FCS+HEP, HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS plus 200 μg/ml HEP; DR+FCS, down-regulated HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS; CHE+FCS, chelerythrine-treated HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS; DR+FCS+HEP, down-regulated HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS plus 200 μg/ml HEP; CHE+FCS+HEP, chelerythrine-treated HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS plus 200 μg/ml HEP; FCS+PMA, HEC stimulated for 1 h (A) or 6 h (C) with 10% FCS plus 100 nM PMA; PMA, HEC stimulated for 1 h (A) or 6 h (C) with 100 nM PMA; PMA+HEP, HEC stimulated for 1 h (A) or 6 h (C) with 100 nM PMA plus 200 μg/ml HEP. Data represent the mean ± S.E. of four different experiments. *, significantly different from untreated. #, significantly different from FCS. §, significantly different from PMA. B, D: dose-response effect of the indicated concentrations of heparin on the PKC-δ (B) and -α (D) mRNA expression induced by 100 nM PMA in HEC. Cells, cultured in serum-free medium, were stimulated for 1 h (B) or 6 h (D) with 100 nM PMA, in the absence (0) or presence of the indicated concentrations of HEP. Expression of PKC-δ (B) and -α (D) mRNA was detected by [α-^{32}P]dCTP-PCR (30 cycles) using 5 μl of RT product. UNTREATED, untreated cells; FCS, HEC stimulated for 1 h (B) or 6 h (D) with 10% FCS; FCS+HEP, HEC stimulated for 1 h (B) or 6 h (D) with 10% FCS plus 200 μg/ml HEP. Data represent the mean ± S.E.M. of four different experiments. *, significantly different from UNTREATED. #, significantly different from FCS. §, significantly different from 0 μg/ml of heparin. Results were normalized to GAPDH mRNA detected in each sample and expressed as a ratio to GAPDH.

that the effect of the polyelectrolyte occurred by the inhibition of a serum-activated PKC-dependent pathway [21]. Here, we investigated whether such a signalling mechanism may also be involved in the induction of PKC genes. Fig. 3 (A, B) shows that in serum-free cells, 100 nM PMA increased the PKC-α and -δ gene expression with a similar time course as that observed in the presence of serum. However, the peak increase in PKC-α or -δ mRNA was higher in serum-stimulated than in PMA-treated cells (Fig. 4A, C) and no additivity was observed between the effects produced by FCS and PMA upon PKC mRNA expression (Fig. 4A, C). The effect elicited by PMA on PKC-α and -δ mRNA levels was dose-dependent (Fig. 3C, E) and was not observed in the presence of chelerythrine or calphostin C, two selective PKC inhibitors [30,31] (Fig. 3D, F). PMA failed to affect the PKC gene expression in PKC down-regulated cells (Fig. 3D, F). The same figure shows that no increase in PKC mRNAs could be observed in cells that were exposed to the biochemically inactive phorbol 4α-phorbol 12, 13-didecanoate (4α-phorbol). On the other hand, both chelerythrine and PKC down-regulation elicited no more than 50% reduction of PKC-α and -δ mRNA in serum-treated cells (Fig. 4A, C). The addition of heparin (200 μg/ml) to chelerythrine-treated or PKC down-regulated cells failed to produce any additive inhibition in the residual effect of FCS (Fig. 4A, C). On the contrary, heparin dose-dependently suppressed the stimulatory effect of PMA on both the PKC-α and -δ gene expression (Fig. 4B, D).

4. Discussion

Heparin and heparan sulfate are complex glycosaminoglycans that exert antiproliferative effects in different cell types [32,33,14–16]. Consonant with growth inhibition, heparin inhibits the expression of the early proto-oncogenes *c-fos* and *c-myc* by acting selectively on a PKC-dependent pathway [18–20]. The inhibition of this signalling pathway was also found to be the mechanism by which heparin down-regulated the ODC gene expression and cell proliferation in EC [21]. It is increasingly becoming evident that PKC isoenzyme gene expression, besides enzyme activity, may elicit complex cellular responses. Overexpression of PKC-α promoted the proliferation of several cell types, including fibroblasts and endothelial cells [34–36], while inhibiting the proliferation of rat smooth muscle cells and F9 teratocarcinoma cell lines [37,38]. Stably transfected NIH 3T3 fibroblasts, overexpressing human PKC-α under the control of the interferon type I inducible murine Mx promoter, exhibited a 10-fold increase in the transcription of PKC-α mRNA along with an increase in the growth rate and early gene expression [39]. Moreover, growth conditions affected the expression of PKC genes. Serum starvation in αT3-1 cells was found to remarkably modify the time course of both phorbol ester- and gonadotropin-releasing hormone A-induced PKC-δ mRNA, as compared to the induction observed in non-starved cells [40].

Here, we assessed whether serum and/or PMA may affect

the PKC gene expression in HEC and hypothesize that the growth inhibitory response elicited by heparin in HEC [21] may be associated with changes in the PKC gene expression. The current experimental data show that the exposure of HEC to serum or the treatment with PMA in a serum-free medium enhanced the expression of both PKC-α and -δ mRNA with a similar time course, suggesting that PKC is involved in serum stimulation of the PKC gene expression. In fact, the treatment with serum in the presence of PMA did not produce additive effects on PKC mRNA levels, further supporting the role of PKC in mediating the serum effect on the PKC gene expression. The possibility that the observed increase in the serum-induced expression of PKC genes may be mediated by the activation of a PKC-dependent pathway is suggested by the finding that chelerythrine and calphostin C, as well as PKC down-regulation, significantly, although not completely, reduced the stimulatory effect of serum. Failure of PKC inhibition or depletion of abolishing completely the serum-induced expression of PKC-α and -δ mRNA indicates that PKC-independent signalling may also be conceivable. In this regard, both PKC-dependent and PKC-independent pathways have been implicated in the ability of serum to promote early gene expression and proliferative responses in VSMC [20]. Consistent with such a possibility are the present observations showing that the peak increase of PKC mRNA levels in PMA-treated cells was significantly lower than that detected in serum-stimulated cells and that PKC inhibition or enzyme down-regulation completely inhibited the PMA-induced PKC gene expression in serum-free cells. These findings are in agreement with other observations indicating that both PKC inhibition and down-regulation where able to completely abolish a stimulatory effect of PKC activation on the PKC-δ [40] and -β gene expression [41]. In addition, in the current investigation, the specificity and PKC-dependence of the stimulatory action of PMA are further supported by the lack of an effect by the inactive phorbol ester 4α-phorbol.

A number of experimental observations in the present study suggest the possibility that heparin might exert its inhibitory action on PKC mRNA levels by inhibiting a PKC-dependent pathway controlling the PKC gene expression. First, heparin only partially decreased the stimulatory effect of serum on PKC-α and -δ mRNA, while completely abolishing the PMA-stimulated PKC gene expression. Secondly, heparin failed to further decrease PKC-α and -δ mRNA levels in chelerythrine-treated serum-supplemented cells. Third, no significant effect of heparin on the PKC gene expression could be observed in serum-exposed cells following PKC down-regulation. These findings support the hypothesis that (i) the polyelectrolyte may have suppressed a serum-activated PKC-dependent pathway controlling the PKC gene expression, being without an effect on a residual serum-stimulated PKC-unrelated gene expression, (ii) PKC inhibition or down-regulation, as well as the treatment in the presence of heparin, might have inhibited the PKC gene expression by following the same signal transduction pathway.

Evidence for a cross-talk between PKC activation and the induction of selected PKC genes has been recently provided. In particular, PKC-α overexpression in Baf3 and 32D cells has been found to elevate the PKC-δ mRNA expression by acting at the transcriptional level. Such an effect was mimicked by phorbol ester and could be abolished by the PKC inhibitor GF109203X, indicating the requirement for PKC activity [42]. The present observation that the inhibitory effect of heparin on the PKC gene expression required an activated PKC-dependent pathway indicates that the polyelectrolyte may abolish some form of cross-regulation between the PKC activity and PKC gene expression. Whether the interruption of this regulatory circuit may affect the distribution of PKC isoenzymes in different subcellular compartments remains to be elucidated.

Similarly, the possible implications of the present study remain to be established. However, it is now evident that the endothelial cell plays a crucial role in the angiogenesis [43] and that it might affect the growth and differentiation of neighboring cells [44]. The endothelial cell is also the target cell for tumor neovascularization [45], a process that closely associates with tumor growth and metastasis [46]. In this regard, heparin, a molecule tightly related to the heparan sulfate component of the extracellular matrix, exerts remarkable effects on the angiogenesis [17] and has been shown to affect the cell proliferation under normal [33,14–16] or pathological conditions [47,48,32]. Interestingly, heparin has been found to inhibit both the increase of the PKC activity and myointimal proliferation elicited by balloon injury in rat carotid artery in vivo [49]. Therefore, heparin-mediated inhibition of PKC gene expression might also represent a vascular remodelling mechanism.

To our knowledge, this is the first report demonstrating that heparin affects the PKC gene expression, through the inhibition of a PKC-related mechanism. The present finding supports the hypothesis that the interruption mediated by heparin of a PKC-dependent control on PKC gene patterning may be a part of the complex machinery by which glycosaminoglycans modulate cellular responses under normal or pathological conditions.

References

[1] Nischizuka, Y. (1992) Science 258, 607–613.
[2] Dekker, L.V. and Parker, P.J. (1994) Trends Biochem. Sci. 19, 73–77.
[3] Azzi, A. and Hensey, C. (1992) Eur. J Biochem. 208, 547–557.
[4] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.
[5] Nischizuka, Y. (1995) FASEB J. 9, 484–496.
[6] Newton, A.C. (1995) J. Biol. Chem. 270, 28495–28498.
[7] Nakanischi, H., Brewer, K.A. and Exton, J.H. (1993) J. Biol. Chem. 268, 13–16.
[8] Kaibuchi, K., Tusda, T., Kikuchi, A., Tanimoto, T., Yamashita, T. and Taka, Y. (1986) J. Biol. Chem. 261, 1887–1892.
[9] Doctrow, S.R. and Folkman, J. (1987) J. Cell. Biol. 104, 679–687.
[10] Kent, K.C., Mii, S., Harrington, E.O., Chang, J.D., Mallette, S. and Ware, J.A. (1995) Circ. Res. 77, 231–238.
[11] Kinsella, J.L.Grant,D.S.Weeks,B.S. and Kleinman, H.K. (1992) Exp. Cell Res. 199, 56–62.
[12] Hu, D.E. and Fan, T.P. (1995) Inflammation 19, 39–54.
[13] Wrigth, P.S., Cross-Doersen, D., Miller, J.A., Jones, W.D. and Bitonti, A.J. (1992) J.. Cell Physiol. 152, 448–457.
[14] Reilly, C.F., Fritze, L.M. and Rosenberg, R.D. (1986) J. Cell Physiol. 129, 11–19.
[15] Rosenbaum, J., Tobelem, G., Molho, P., Barzu, T. and Caen, J.P. (1986) Cell Biol. Int. Rep. 10, 437–446.
[16] Kimura, I., Nagaura, T., Naitoh, T., Kobayashi, S. and Kimura, M. (1992) Jpn. J. Pharmacol. 60, 369–375.
[17] Hahnenberger, R., Jakobson, A.M., Ansari, A., Wehler, T., Svahn, C.M. and Lindahl, U. (1993) Glycobiology 3, 567–573.
[18] Wright Jr., T.C., Pukac, L.A., Castellot Jr., J.J., Karnovsky,

M.J., Levine, R.A., Kim-Park, H.Y. and Campisi, J. (1989) Proc. Natl. Acad. Sci. USA 86, 3199–3203.
[19] Castellot Jr., J.J., Pukac, L.A., Caleb, B.L., Wright Jr., T.C. and Karnovsky, M.J. (1989) J. Cell Biol. 109, 3147–3155.
[20] Pukac, L.A., Ottlinger, M.E. and Karnovsky, M.J. (1992) J. Biol. Chem. 267, 3707–3711.
[21] Pintus, G., Tadolini, B., Maioli, M., Posadino, A.M., Bennardini, F., Bettuzzi, S. and Ventura, C. (1998) FEBS Lett. 423, 98–104.
[22] Jaffe, A.E., Nachman, L.R., Becker, G.C. and Minick, R.C. (1973) J. Clin. Invest. 52, 2745–2756.
[23] Kent, K.C., Collins, L.J., Schweirn, F.T., Raychowdhury, M.K. and Ware, J.A. (1993) Circ. Res. 72, 958–965.
[24] Zhou, W., Takuwa, N., Kumada, M. and Takuwa, Y. (1993) J. Biol. Chem. 268, 23041–23048.
[25] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[26] Rappolee, D.A., Mark, D., Banda, M.J. and Werb, Z. (1988) Science 241, 708–712.
[27] Singer-Sam, J., Robinson, M.O., Bellve, A.R., Simon, M.I. and Riggs, A.D. (1990) Nucleid Acids Res. 18, 1255–1259.
[28] Li, J.M. and Brooks, G. (1997) Am. J. Physiol. 273, H1358–H1367.
[29] Webb, B.L., Lindsay, M.A., Seybold, J., Brand, N.J., Yacoub, M.H., Haddad, E.B., Barnes, P.J., Adcock, I.M. and Giembycz, M.A. (1997) Biochem. Pharmacol. 54, 199–205.
[30] Herbert, J.M., Augereau, J.M., Gleye, J. and Maffrand, J.P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999.
[31] Tamaoki, T. (1991) Methods Enzymol. 201, 340–347.
[32] Floege, J., Eng, E., Young, B.A., Couser, W.G. and Johnson, R.J. (1993) Kidney Int. 43, 369–380.
[33] Ferrao, A.V. and Mason, R.M. (1993) Biochim. Biophys. Acta 1180, 225–230.
[34] Harrington, E.O., Loffer, J., Nelson, P.R., Kent, C.K., Simons, M. and Ware, A.J. (1997) J. Biol. Chem. 272, 7390–7397.
[35] Eldar, H., Zisman, Y., Ullrich, A. and Livneh, E. (1990) J. Biol. Chem. 265, 13290–13296.
[36] Ways, D.K., Kukoly, C.A., deVente, J., Hooker, J.L., Bryant, W.O., Posekany, K.J., Fletcher, D.J., Cook, P.P. and Parker, P.J. (1995) J. Clin. Invest. 95, 1906–1915.
[37] Kindregan, H.C., Rosenbaum, S.E., Ohno, S. and Niles, R.M. (1994) J. Biol. Chem. 269, 27756–27761.
[38] Wang, S., Desai, D., Wright, G., Niles, R.M. and Wright, G.L. (1997) Exp. Cell. Res. 236, 117–126.
[39] Finkenzeller, G., Marme, D. and Hug, H. (1992) Cell Signal 4, 163–177.
[40] Harris, D., Reiss, N. and Naor, Z. (1997) J. Biol. Chem. 272, 13534–13540.
[41] Shraga-Levine, Z., Ben-Menahem, D. and Naor, Z. (1994) J. Biol. Chem. 269, 31028–31033.
[42] Romanova, L.Y., Alexandrov, I.A., Nordan, R.P., Blagosklonny, M.V. and Mushinski, J.F. (1998) Biochemestry 37, 5558–5565.
[43] DeLisser, H.M., Christofidou-Solomidou, M., Strieter, R.M., Burdick, M.D., Robinson, C.S., Wexler, R.S., Kerr, J.S., Garlanda, C., Merwin, J.R., Madri, J.A. and Albelda, S.M. (1997) Am. J. Pathol. 151, 671–677.
[44] Bobik, A. and Campbel, H.J. (1993) Pharmacol. Rev. 45, 1–42.
[45] Murray, J.C., Hewett, P.W. and Martin, S.G. (1996) Q. J. Med. 89, 165–167.
[46] Stetler-Stevenson, W.G. and Corcoran, M.L. (1997) EXS 79, 413–418.
[47] Bertolesi, G.E., Lauria de Cidre, L. and Eijan, A.M. (1994) Tumor Biol. 15, 275–283.
[48] Chan, P., Mill, S., Mulloy, B., Kakkar, V. and Demoliou-Mason, C. (1992) Int. Angiol. 11, 261–267.
[49] Herbert, J.M., Clowes, M., Lea, H.J., Pascal, M. and Clowes, A.W. (1996) J. Biol. Chem. 271, 25928–25935.