ELSEVIER

FEBS Letters 342 (1994) 76–80

FEBS 13850

FEBS LETTERS

Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation

Jean W. Assender[a,b,*], Ewa Kontny[a,c,**], Bertil B. Fredholm[a]

[a]*Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77, Stockholm, Sweden*
[b]*Department of Cardiology, University of Wales College of Medicine, Cardiff, UK*

Received 14 February 1994

Abstract

We analysed the expression of protein kinase C (PKC) isoforms in smooth muscle cells (SMC) in various states of differentiation, using Western blots and thermocycle amplification of mRNA. SMC isolated from intact tissues express three isoforms of PKC, namely α, δ, and ζ. Following cell culture, SMC additionally express mRNA for PKC-ε, but significant amounts of the corresponding protein were not detected. Transformed SMC, such as the cell line DDT_1 MF-2, express both mRNA and protein for PKC-ε, lack the δ isoenzyme, whilst maintaining the expression of the α and ζ isoforms. Thus PKC-δ and ε isoenzyme expression appears to vary with the state of differentiation of these cells, with PKC-ε expression increasing as the cells become proliferative.

Key words: Protein kinase C; Differentiation; Smooth Muscle Cell

1. Introduction

Extracellular signals can activate the phospholipid-dependent protein kinase C (PKC) by phospholipase C (PLC)-derived *sn*-1,2-diacylglycerol (DAG), by DAG derived from phosphatidylcholine via the actions of phosholipase D and phosphatidic acid phosphohydrolase, by arachidonic acid and by unsaturated fatty acids [1]. PKC also acts as the cellular receptor for the tumour promoting phorbol esters [2].

There are at least 8 distinct isoforms of this enzyme, which can be divided into two classes; the classical, calcium-dependent isoforms (α, β and γ), and calcium-independent forms (δ, ε, η and ζ) [3,4]. All PKC isoforms are present in the brain, whereas other tissues appear to express a limited repertoire, with certain isoforms more widely distributed than others [4,5]. Although the isoforms appear to be associated with different functions [6,7], their roles in specific cells have yet to be determined.

Smooth muscle cell (SMC) proliferation is clinically important in e.g. atherosclerosis, angioplasty, re-stenosis and vein graft occlusion. According to the 'response to injury' hypothesis [8] this proliferation is the result of locally released growth factors. Growth factors bind to cell surface receptors, leading to tyrosine kinase activation, receptor autophosphorylation and activation of a kinase cascade thought to involve Ras protein, Raf-1, mitogen-activated protein (MAP) kinase kinase and MAP kinase [9,10]. Stimulation of G-protein coupled receptors, e.g. by serotonin or endothelin, can also lead to proliferation [11]. The mechanism is less clear, but may involve PKC. Proliferating cells exhibit greater PKC activity than quiescent cells, and more of the is enzyme associated with the particulate fraction [12]. Also, direct activation of PKC by phorbol esters may induce SMC proliferation, whilst inhibition of PKC by selective inhibitors can inhibit it [13].

Previous observations suggested that SMC express at least 2 isoforms of PKC [14], and that these may have distinct functions with regard to regulating SMC proliferation [15]. Since the state of differentiation of SMC is altered in the neointimal thickenings of injured or atherosclerotic arteries compared to those in the quiescent, contractile artery, we studied PKC isoform expression in cells from various sources. The results suggest that PKC expression depends on SMC differentiation.

*Corresponding author. Fax: (46) (8) 33 16 53.

***Present address:* Instytut Reumatologiczny, 02 - 637 Warszawa, Spartanska 1, Poland.

Abbreviations: PKC, protein kinase C; SMC, smooth muscle cell; TPA, 12-*O*-tetradecanyl-phorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; FCS, fetal calf serum.

SSDI 0014-5793(94)00235-N

2. Materials and methods

2.1. Materials

Cultured human renal artery SMC were a gift from Dr. J. Nilsson at the vascular cell biology group at the Karolinska Hospital, Stockholm. DDT_1 MF-2 SMC derived from steroid-induced leiomyosarcoma of Syrian hamster vas deferens [16], were purchased from ATCC. Human saphenous vein was obtained from the Dept. Cardiology, Cardiff. Rat vas deferens were freshly dissected out. Antibodies to PKC α, β_c, β_1, β_{II}, γ and ε and the corresponding purified enzymes were kindly provided by Prof. Nishizuka, Kobe University, Japan; antibodies to PKC δ, ε and ζ and corresponding peptides were purchased from Gibco (BRL, Grand Island, NY, USA). All the antibodies were raised against the $CKpV_5$ region except those to γ ($CKpV_3$) and to the common region of the β isoforms (β_c, $CKpV_1$). Molecular biology reagents were either from Promega (Scandinavian Diagnostic Services (SDS), Falkenberg, Sweden) or Pharmacia-LKB (Uppsala, Sweden).

2.2. SMC Culture

SMC were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with penicillin/streptomycin, L-glutamine and fetal calf serum (FCS, 10%), in a humidified 5% CO_2/95% O_2 atmosphere, at 37°C. Cells were passaged using trypsin (0.05%)-EDTA (0.02% w/v in normal saline) as required and batches frozen in liquid nitrogen. Two time-points for analysis were selected – namely after passage 6–8 or after passage 27–30 – to differentiate between cell populations containing some 'reversibly' synthetic cells, and SMC selected by multiple passage for their synthetic ability. All cell populations were confirmed to be pure cultures of SMC by positive staining with a monoclonal antibody to α-SMC actin (Clone 1A4, Sigma).

DDT_1 MF-2 cells were cultured under similar conditions except that only 5% FCS was required.

2.3. Protein analysis by Western blotting

SMC (5×10^5 cells) were plated onto 10 mm diameter Petri dishes and grown for 2 days. Cellular protein was extracted by washing the cells with phosphate-buffered saline (PBS) and then treating with 1.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) supplemented with 0.05 mg/ml digitonin, 0.1% Triton X-100, 10 μg/ml leupeptin and 10 μg/ml pepstatin A. After 5 min on ice, the cells were scraped into Eppendorff tubes and vortexed (30 min, 4°C). The supernatant after centifugation ($12,000 \times g$, 15 min) was acetone precipitated at −20°C overnight. The resulting protein pellet was resuspended in Laemmli sample buffer, boiled for 5 min and then run on an 8% polyacrylamide, denaturing gel.

DDT_1 MF-2 cells (10^6 cells/sample) were pelleted by centrifugation ($5,000 \times g$, 5 min), washed in PBS, resuspended in lysis buffer and proteins extracted as above. Protein from intact vessels was prepared by crushing the vessel under liquid nitrogen and then rapidly resuspending the powder in boiling Laemmli sample buffer. Rat brain cortex and vas deferens were homogenized in lysis buffer and then sonicated for 2 min. The supernatant after centrifugation (1 h, $100,000 \times g$, 4°C) was acetone precipitated, resuspended in Laemmli buffer and electrophoresed, as above.

Proteins were blotted onto a membrane (Immobilon, Millipore, Bedford, MA, USA) and non-specific binding blocked by an overnight incubation in Tris-buffered saline (TBS; Tris-HCl, pH 7.6, 500 mM NaCl) containing Tween-20 (0.5%) and 5% FCS. Membranes were incubated with PKC-specific antibodies for 3 h, before washing 3×10 min with TBS-Tween. Antibody binding was detected with a second antibody (horseradish peroxidase conjugated anti-rabbit), and an enhanced chemiluminescence system (Amersham, GB). Specificity of the primary antibody binding was checked by preincubation of the antibody with isoform-specific peptide (0.5 mg/ml) or purified enzyme (2 μg/ml) for 10 min at room temperature prior to use.

2.4. TPA treatment and cell fractionation

SMC were grown in Petri dishes as above, but prior to protein extraction, the cells were washed with DMEM containing 20 mM HEPES and then reacted with 12-*O*-tetradecanyl-phorbol 13-acetate (TPA, 100 nM) for 10 min, 37°C. The cells were then washed with PBS and treated with lysis buffer supplemented as above but lacking Triton X-100. The protein suspension was centrifuged ($12,000 \times g$, 10 min), the supernatant removed and the proteins were acetone precipitated (the 'cytosolic' fraction). The membrane pellet was resuspended in lysis buffer containing 0.1% Triton X-100 (400 μl) and vortex mixed for 30 min. Centrifugation ($12,000 \times g$, 10 min) yielded a supernatant containing the 'membrane fraction' and a pellet referred to as the 'urea fraction'. This pellet was vortexed with 8 M urea in 10% SDS, for 20 min, centrifuged ($12,000 \times g$, 10 min), and the supernatant remaining was acetone precipitated. Proteins in the 'membrane fraction' were also acetone precipitated overnight at −20°C. The protein was collected by centrifugation ($12,000 \times g$, 15 min), resuspended in Laemmli buffer and the samples run on SDS-PAGE and blotted as described above.

2.5. Thermocycle analysis of mRNA

Cytoplasmic RNA from either SMC, DDT_1 MF-2 cells or cerebellum, was prepared using a 'mini-prep' method [17] and messenger (m)RNA selected using an oligo d(T) column. Vascular tissue was crushed under liquid nitrogen, resuspended in Solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 100 mM β-mercaptoethanol), DNA sheared through a needle, and the RNA was phenol/chloroform extracted. Remaining DNA contamination was removed by DNAase digestion (37°C, 40 min) and mRNA selected using an oligo d(T) column. Vas deferens was crushed under liquid nitrogen and mRNA extracted using the PolyAT tract system 1000 (Promega, SDS, Sweden).

cDNA was produced from approximately 0.06 μg mRNA, using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) 10 units, 40°C for 50 min, in a buffer containing 6.9 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 4 mM dNTP, 1 unit rRNasin ribonuclease inhibitor, 19 μM $pd(N)_6$ random hexamers, final volume 20 μl. This cDNA was then analysed by thermocycle amplification of sequences specific for the various isoforms of PKC. The sequences amplified were based on ones previously selected by Freire-Moar et al. [18] i.e. those sequences between base pairs 875–1199 for α, 925–1188 for β, 713–1257 for γ, 1013–1364 for δ, 577–1308 for ε, and 404–1085 for ζ. Amplification of 10 μl cDNA was performed with 0.025 units *Thermus aquaticus Taq* DNA polymerase in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 5.4 mM $MgCl_2$, 0.9 mM dNTP, and 2.8 pg of each primer, final volume 50 μl. PKC isoforms α and δ were run on a thermoamplification program consisting of an initial denaturation at 94°C, 5 min, followed by 35 cycles of 1 min denaturation (92°C), 2 min annealing (55°C) and 2 min elongation (72°C), with a final extension period of 5 min at 72°C. The longer products (γ, ε and ζ) were run on the same program except that the length of the elongation period was increased to 3 min. The β isoform however required a different protocol: 1 min denaturation (94°C), 14 cycles of 1 min 95°C, 2 min 55°C, 1 min 72°C, then 19 cycles of 1 min 95°C, 2 min 55°C, 3 min 72 °C, followed by 5 min at 72°C. Products were run though a 2% agarose gel, containing 3% (v/v) ethidium bromide and detected by trans-illumination with UV light.

3. Results and discussion

Western blot analysis demonstrated the presence of three isoforms of PKC in SMC, namely PKC-α, δ and trace amounts of ζ (Fig. 1). Rat brain protein was run as a positive control and clearly shows that under the conditions employed all 7 isoforms of PKC studied could be detected (Fig. 1). The molecular weights of these proteins were consistent with previously published findings [3,5,19]. As further confirmation that the proteins detected were PKC, the primary antibody was preincubated with the corresponding purified enzyme or with the peptide against which the antibody was raised, and this was shown to prevent protein detection (Fig. 2).

The type III enzyme previously described [14] as having both proliferative and anti-proliferative actions in SMC, was probably PKC-α. Consistent with the earlier

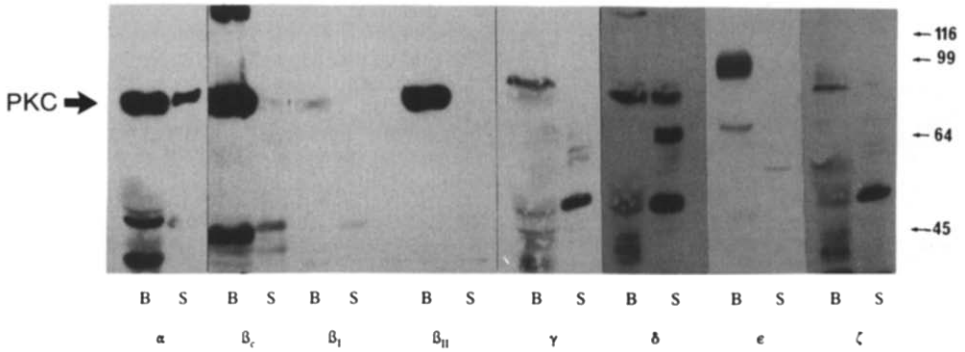

Fig. 1. Protein kinase C (PKC) isoform expression examined by Western blot. Smooth muscle cell (S) protein was compared with a rat brain (B) positive control.

observations, we found it to be readily down-regulated by long-term treatment with phorbol ester (Fig. 2).

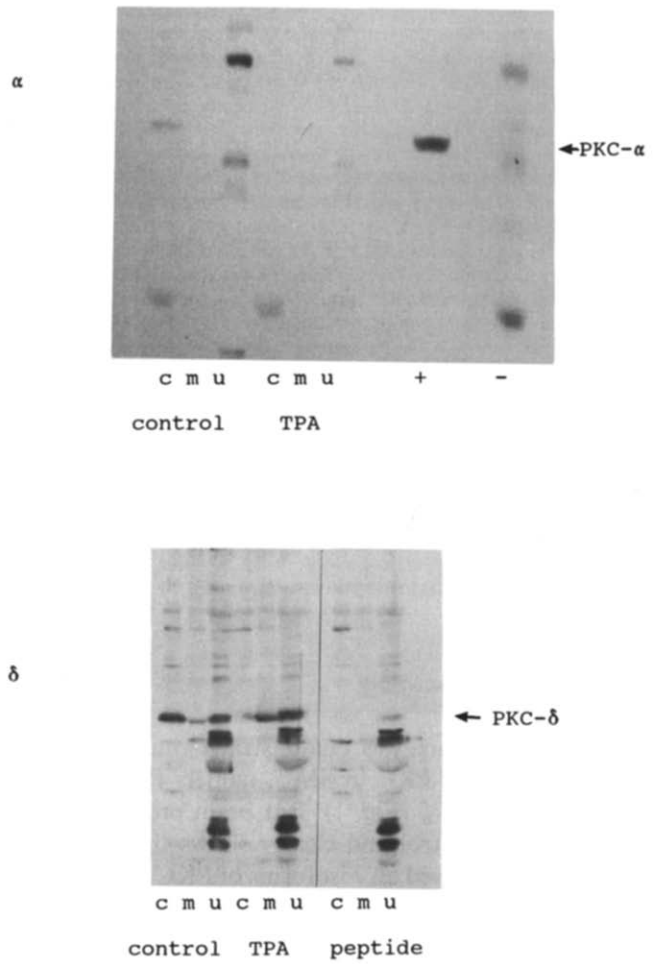

Fig. 2. Activation and translocation of protein kinase C (PKC) by phorbol ester (TPA). (Upper panel) PKC-α detected in rat brain positive control (+), blocked by preincubation of the antibody with PKC-α peptide (−), and in smooth muscle cells lysed to produce cytosolic (c), membrane (m) and urea extractable (u) fractions. Upon stimulation with TPA (100 nM, 10 min) PKC-α was lost from the c fraction. (Lower panel) PKC-δ detection in fractionated unstimulated smooth muscle cells (control) or following TPA stimulation. Preincubation of the antibody with δ peptide greatly reduced the signal detected in all fractions.

PKC-δ appeared to be present in abundance in SMC, with a large proportion of this isoform associated with the membrane fraction even in unstimulated cells. The tight nature of this association, possibly involving intergration into a membrane, was illustrated by the finding that treatment with 8 M urea was required to fully dissociate it. Treatment of the cells with TPA for 10 min, lead to a further translocation of PKC-δ to the membrane fraction (Fig. 2).

Thermocycle analysis of cultured SMC mRNA confirmed that these cells express PKC isoforms α and δ, but in additon, PKC-ε was also clearly detected (Fig. 3). In the absence of either mRNA or of reverse transcriptase enzyme no band was detected, indicating that the samples were not contaminated with exogenous or endogenous DNA. Isoform expression appeared to be the same in both early (6–8th) and late (27–30th) passage SMC (Fig. 4). Comparison with the protein analysis, suggests that mRNA for PKC-ε is expressed in cultured SMC without significant translation into protein. Lack of antibody immunoreactivity to the human sequence is unlikely to account for the lack of ε protein detection, since ε protein also could not be detected even in rabbit aorta, despite rabbit being a species to which the antibody is known to react. In addition, neither the Nishizuka nor the Gibco antibody detected the protein, despite reacting to slightly different epitopes.

Little if any mRNA for PKC-ζ could be detected in cultured SMC. This was perhaps due to the fact that prior to RNA extraction the cells were grown to confluency and had began to quiesce. If mRNA was extracted from SMC which were actively proliferating, some PKC-ζ could be detected (data not shown). The absence of isoforms β and γ from these cells was confirmed by both Western blot and molecular analysis; the enzyme could not be detected using conditions which allowed the detection of a positive (brain) control. This pattern of isoform expression is again consistent with that previously reported [14] for SMC. In addition, renal mesangial cells have been found to express only PKC-α, δ, ε and ζ [20], consistent with the great similarity known

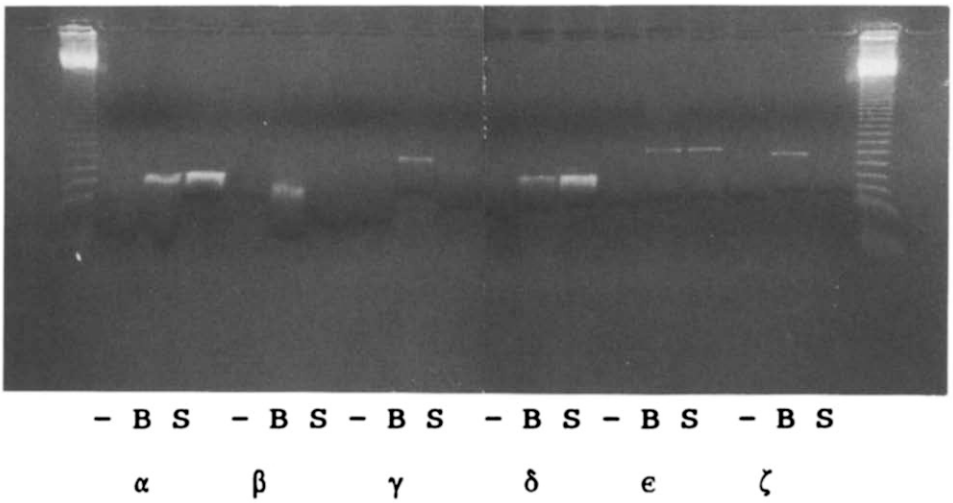

Fig. 3. Molecular analysis of protein kinase C (PKC) isoforms in smooth muscle cells (S). Rat cerebellum (B) was used as a positive control, RNA minus the reverse transcriptase enzyme or reaction mix minus RNA was run as a negative control (−).

to exist between these cell types. In contrast, cardiac myocytes express PKC-β [21], a difference possibly related to the differences in signalling pathways seen between these cells. Lack of PKC-γ expression was expected since this isoform is mainly located in neuronal tissues, although it has been reported to be present in some leukemic and glioblastoma cell lines [22,23].

Contractile SMC from intact vessels (human saphenous vein and rabbit aorta) were analysed and showed a similar pattern of protein staining to cultured SMC, i.e. isoforms α, δ and some ζ could be detected (Fig. 5.1). Analysis of mRNA showed that, unlike their proliferating counterparts, contractile SMC do not express PKC-ε mRNA (Fig. 4). Next, cells of the smooth muscle tumour cell line DDT_1 MF-2 were studied. PKC-α was expressed together with significant amounts of PKC-ζ. PKC-δ was absent; however, PKC-ε could be detected both by Western blotting (Fig. 5.3) and by molecular analysis (Fig. 4). To test the trivial explanation that DDT_1 MF-2 cells are derived from vas deferens rather than vascular tissue, PKC expression in freshly isolated vas deferens was examined. However, the pattern of PKC expression was the same as in blood vessels, with mRNA and protein detectable for PKC-α, δ and ζ, but not for ε (Fig. 5.2 and 6). The results therefore suggest that PKC-δ and -ε expression alters with the state of differentiation of SMC, not their source: no ε-isoform was detected in contractile cells. mRNA was expressed in cultured SMC but not translated to protein, whilst transformed SMC both transcribe and translate PKC-ε.

PKC-α, was expressed in SMC in all states of differentiation, consistent with the belief that this ubiquitous isoform serves a general role in cell physiology. It is interesting to note that despite the observed modification in ε-isoform expression, SMC always expressed both a calcium-dependent and a calcium-independent isoform of PKC. Further it is interesting to speculate on the role of PKC-δ. The tight association with the membrane frac-

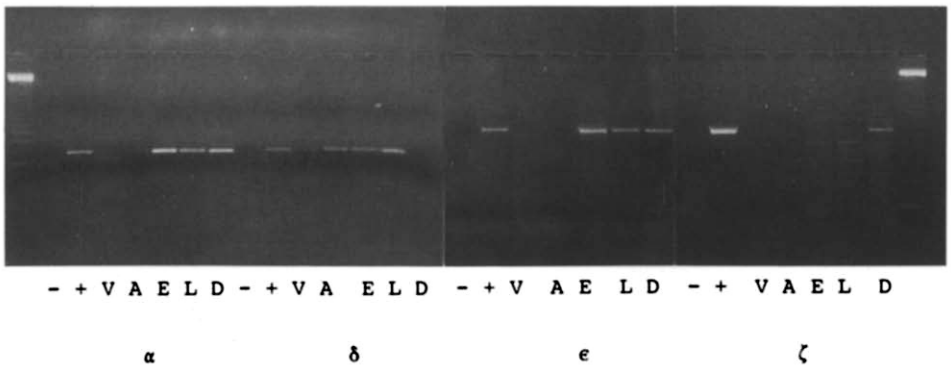

Fig. 4. Investigation of protein kinase C isoform expression by smooth muscle cells (SMC) in various states of differentiation. Samples were analysed by thermocycle amplification. A cerebellum positive (+) control and a negative (−) minus RNA or minus reverse transcriptase control was run. Contractile SMC from human saphenous vein (V) or rabbit aorta (A) were compared with cultured SMC from early (E, 6–8th) or late (L, 27–30th) passage, and with a SMC line DDT_1 MF-2 cells (D).

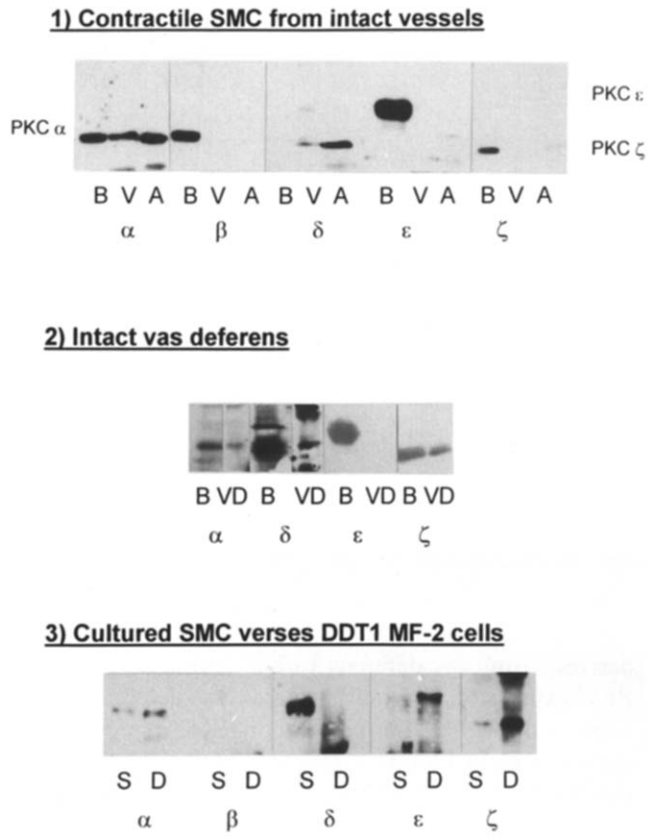

Fig. 5. Western blot analysis of protein kinase C isoenzyme expression in different tissues. (1) Comparison of human saphenous vein (V), rabbit aorta (A) and cerebellum positive control (B). (2) Intact vas deferens (VD). (3) Cultured vascular SMC (S) and the vas deferens derived tumour SMC line DDT_1 MF-2 cells (D).

tion and its expression in contractile state cells, may indicate a role in cell contractility. In addition, PKC-δ has previously been shown to inhibit cell-cycle progression by blocking cytokinesis [24] indicating a possible role for this isoform in SMC growth inhibition.

In conclusion, we have demonstrated the presence of four isoforms of PKC in SMC. PKC-α is generally expressed as is PKC-ζ. The δ isoform is found in intact vessels and cultured cells but is lost from cells during transformation. The expression of PKC-ε appears to increase with the state of dedifferentiation of the cells, in accordance with the observation [25] that down-regulation of PKC-ε promotes neuroblastoma cell differentiation.

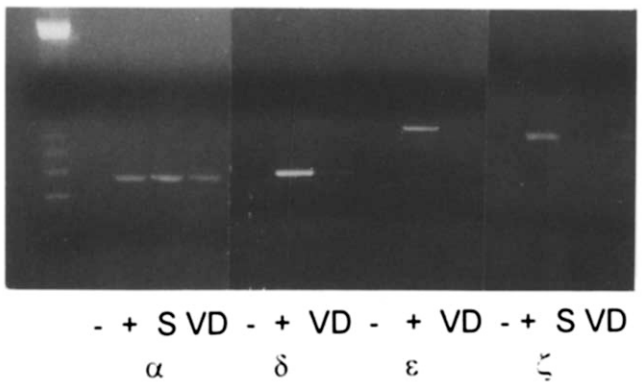

Fig. 6. Expression of protein kinase C (PKC) isoform mRNA in vas deferens. A cerebellum positive control (+), mRNA from cultured SMC (S) and from intact vas deferens (VD) were run. Lack of RNA or reverse transcriptase enzyme was used as a negative control (−).

Acknowledgements: We thank Prof. Nishizuka for providing us with antibodies. This work was supported by grants from the Swedish Cancer Fund, Gustaf V's 80 years Fund, Nanna Svartz' Fund and the Karolinska Institutet. J.A. was a recipient of grants from the Wenner-Gren Foundation and the British Heart Foundation.

References

[1] Nishizuka, Y. (1986) Science 233, 305–312.
[2] Sharkey, N.A. (1984) Proc. Natl. Acad. Sci. USA 81, 607–610.
[3] Ono, Y., Fujii, T., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
[4] Osada, S.I., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) J. Biol. Chem. 265, 22434–22440.
[5] Ohno, S., Kawasaki, H., Imajoh, S., Sukuki, K., Inagaki, M., Yokokura, H., Sakoh, T. and Hidaka, H. (1987) Nature 325, 161–166.
[6] Ozawa, K., Szallasi, Z., Kazanietz, M.G., Blumberg, P.M., Mischak, H., Mushiski, J.F. and Beaven, M.A. (1993) J. Biol. Chem. 268, 1749–1756.
[7] Ozawa, K., Yamada, K., Kazanietz, M.G., Blumberg, P.M. and Beaven, M.A. (1993) J. Biol. Chem. 268, 2280–2283.
[8] Ross, R. (1986) N. Engl. J. Med. 314, 488–500.
[9] Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Science 260, 1658–1661.
[10] Schlessinger, J. and Ullrich, A. (1992) Neuron 9, 383–391.
[11] Simonson, M.S. and Herman, W.H. (1993) J. Biol. Chem. 268, 9347–9357.
[12] Adamo, S., Caporale, C., Aguanno, S., Lazdins, J., Faggioni, A., Belli, L., Cortesi, E., Nervi, C., Gastaldi, R. and Molinaro, M. (1986) FEBS Lett. 195, 352–356.
[13] Newby, A.C., Assender, J.W., Evans, M.A., Lim, K., Bennett, M.R. and Evans, G.I. (1994) Exp. Nephrol. 79, in press.
[14] Kariya, K.I., Kawahara, Y., Fukuzaki, H., Hagiwara, M., Hidaka, H., Fukumoto, Y. and Takai, Y. (1989) Biochem. Biophys. Res. Commun. 161, 1020–1027.
[15] Kariya, K.I. and Takai, Y. (1987) FEBS Lett. 219, 119–124.
[16] Norris, J.S., Gorski, J. and Kohler, P.O. (1974) Nature 248, 422–424.
[17] Wilkinson, M. (1988) Nucleic Acids Res. 16, 10933.
[18] Freire-Moar, J., Cherwinski, H., Huang, F., Ransom, J., and Webb, D. (1991) J. Immunol. 147, 405–409.
[19] Nakanishi, H. and Exton, J.H. (1992) J. Biol. Chem. 267, 16347–16354.
[20] Huwiler, A., Fabbro, D., Stabel, S. and Pfeilschifter, J. (1992) FEBS Lett. 300, 259–262.
[21] Kariya, K.-I., Karns, L.R. and Simpson, P.C. (1991) J. Biol. Chem. 266, 10023–10026.
[22] Komada, F., Nishikawa, M., Uemura, Y., Morita, K., Hidaka, H. and Shirakawa, S. (1991) Cancer Res. 51, 4271–4278.
[23] Misra-Press, A., Fields, A.P., Samols, D. and Goldthwait, D.A. (1992) Glia 6, 188–197.
[24] Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. USA 89, 10159–10163.
[25] Leli, U., Parker, P.J. and Shea, T.B. (1992) FEBS Lett. 297, 91–94.