Regulation of protein kinase C in the muscular layer of human placental stem villi vessels

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Abstract Protein kinase C (PKC) activity in the muscular layer of stem villi vessels from the human term placenta was studied. Resting state PKC activity was distributed evenly between the cytosol and the particulate fractions. Upon stimulation by three different activators, phorbol 12-myristate 13-acetate, fluoride and endothelin-1, a translocation of PKC activity from the cytosolic to the particulate fraction was observed. The expression and distribution of PKC isoforms were then examined by Western blot analysis using specific antibodies to PKC isoforms. At least four PKC isoforms, PKCα, PKCβ$_1$, PKCβ$_2$, PKCζ, and trace amounts of PKCε were detected in both fractions. Their relative responses to the different agonists were examined by quantifying their subcellular redistribution. No significant differential activation of the four mainly expressed PKC isoforms were observed in response to stimulation with any of the stimuli. Moreover, our results show that endothelin-1 induced translocation/activation of PKC in this vascular smooth muscle.

Key words: Protein kinase C; Isoform; Endothelin-1; Translocation; Placental villi vessel

1. Introduction

Protein kinase C (PKC) plays a fundamental role in the regulation of many signal transduction mechanisms activated in response to a variety of stimuli (hormones, growth factors, neurotransmitters). Molecular cloning and biochemical studies have revealed that this kinase consists of a family of at least 12 closely related isoforms classified into four groups based on their primary structure and cofactor requirements. The conventional cPKCs (α, β$_1$, β$_2$ and γ) are Ca^{2+}-dependent and are activated by diacylglycerol (DAG) and phorbol esters. The novel nPKCs (δ, ε, η and θ) are Ca^{2+}-independent and require DAG or phorbol ester for activation. The atypical aPKCs (ζ, λ, ι) are Ca^{2+}-independent and are not activated by DAG or by phorbol esters. A Ca^{2+}-independent isoform, PKCμ, has recently been identified. The patterns of their intracellular and tissue distributions suggest that each PKC isoform regulates specific cellular pathways leading to specific responses [1]. PKC isoforms are present in all tissues to varying degrees. Some isoforms are ubiquitous (PKCα, PKCζ), while others are present in only a few tissues.

PKC appears to perform a variety of functions in vascular smooth muscle. Many studies have reported that the activation of PKC is associated with vascular smooth muscle contractility and plays a major role in growth-related signal transduction [2].

The feto-placental circulation provides for the metabolic needs of the fetus, and regulation of blood flow in this system is critical for fetal well-being and normal development. Stem villi vessels are considered to be the major sites of fetal placenta vascular resistance [3]. Since the placental vessels lack autonomic innervation, vascular tone is regulated by locally or humorally delivered vasoactive substances [4]. Endothelin-1 (ET-1), a 21 amino acid peptide, is a potent vasoactive agent that acts on the contractility of placental vessels [5]. Several studies have reported that activation of PKC may be a component of the signal cascade resulting in the effects of this peptide on contractility and cell division in vascular smooth muscles, such as rat cardiomyocytes [6–8], bovine cerebral arteries [9], human and rat renal artery [10,11], rat aorta [12] and the rat portal vein [13]. Specific high affinity binding sites for ET-1 have been described in the muscular layer of stem villi vessels [14], and Mondon et al. [15] demonstrated that these ET-1 vascular binding sites are coupled to a phosphoinositide-specific phospholipase C pathway that generates two intracellular messengers, DAG and Ca^{2+}, that are activators of PKC.

The objective of this study was to examine the presence of PKC activity in the muscular layer of human placental stem villi vessels. The expression and distribution of PKC isoforms were then determined by Western blot analysis using specific antibodies to PKC isoforms. Activation of PKC by three different stimuli, ET-1, a phosphoinositide-coupled agonist, fluoride, a G-protein activator, and phorbol 12-myristate 13-acetate, a direct activator, was assessed by measuring its translocation from the cytosolic to the particulate fractions. For this purpose, two approaches were used: the redistribution of PKC enzyme activity measured by phosphorylation of an exogenous substrate, and an increase in particulate-associated PKC immunoreactivity determined by Western blot analysis.

2. Materials and methods

2.1. Chemicals

[γ^{32}-P]ATP (3000 Ci/mmol), Hybond-C membranes, the enhanced chemiluminescence detection system (ECL) and X-ray films were obtained from Amersham International (Buckinghamshire, UK). Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4αPDD), leupeptin, Nonidet P-40, histone H1 (type III S), phosphatidylserine, 1,3-diolein, phenylmethylsulfonyl fluoride (PMSF) and other drugs and chemicals were of the highest quality available from Sigma (St, Louis, MO, USA).

Antibodies against PKCα, PKCδ, PKCε and PKCζ were from Gibco BRL (Cergy Pontoise, France). They were raised in rabbits against peptides 313–326 from PKCα, 662–673 from PKCδ, 726–737 from PKCε and 577–592 from PKCζ. Antibodies against PKCβ$_1$

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and PKCβ_2 were from Santa Cruz Biotechnology Inc. (Le Perray en Yvelines, France). They were raised in rabbits against peptides corresponding to amino acid sequences 656–671 and 657–673. The secondary antibody, donkey anti-rabbit IgG conjugated to horseradish peroxidase, was purchased from Amersham. Endothelin-1 (ET-1) was supplied by Neosystem Laboratoire (Strasbourg, France). DEAE-cellulose (DE-52) was obtained from Whatman and prestained molecular weight markers were from Bio-Rad.

2.2. Placenta

Human placentas were obtained from normal term pregnancies immediately after vaginal delivery. Placental tissue was excised between the basal and chorionic plates and stem villi vessels were dissected as reported previously [15].

2.3. Stimulation and preparation of subcellular fractions

Samples of stem villi vessel smooth muscle (150–200 mg) were placed in 1.5–2 ml Krebs Ringer buffer (KRB) containing 12 mM glucose and incubated at 37°C for 15 min under an atmosphere of 95% O_2-5% CO_2. The effectors were then added for various times and the reactions were stopped by cooling to 4°C. Enzyme was extracted by homogenizing the tissue in 5 volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM EGTA, 2 mM EDTA, 50 mM mercaptoethanol, 2 mM PMSF plus 5% glycerol and 20 μg/ml leupeptin using an Ultra-Turrax apparatus. The homogenate was centrifuged at $1000 \times g$ for 15 min to remove debris and nuclei; the supernatant was then ultracentrifuged for 60 min at $100\,000 \times g$. The resulting supernatant (cytosol) was removed and stored at 4°C. The pellet was resuspended in the homogenization medium containing 1% Nonidet P-40, gently mixed for 45 min on ice and centrifuged at $100\,000 \times g$ for 30 min. The resulting supernatant was the solubilized particulate PKC fraction.

Protein kinase C was partially purified from the cytosolic and particulate fractions by DEAE-cellulose chromatography. Samples were loaded onto DE-52 columns (5 ml bed volume) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10 mM mercaptoethanol, 5% glycerol and 20 μg/ml leupeptin. The columns were washed with 3 volumes of the same buffer and PKC was eluted stepwise with buffer containing 120 and 200 mM NaCl. Fractions of 1 ml were collected and assayed for PKC activity.

2.4. Protein kinase C activity assay

Protein kinase C activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone III S. Aliquots of each sample (50 μl) were incubated in a reaction mixture (final volume 250 μl) containing 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 100 μM ATP, 2 μCi/ml [γ-^{32}P]ATP, 0.6 mM Ca^{2+}, 160 μg/ml histone III S, 20 μg/ml phosphatidylserine and 3 μg/ml 1,2-diolein. The reaction was initiated by adding [γ-^{32}P]ATP. Each fraction was incubated without phospholipids and Ca^{2+} but with 1 mM EGTA for 5 min at 30°C. Incubation was stopped by adding 1 ml 20% ice-cold (w/v) trichloroacetic acid (TCA) in 10 mM potassium monophosphate followed by 500 μg of BSA added as carrier. The mixture was centrifuged ($2500 \times g$, 15 min) and the resulting pellet was solubilized with 1 N NaOH and precipitated again with TCA. This procedure was repeated twice. Finally, the pellet was dissolved in 1 N NaOH and its radioactivity was counted by liquid scintillation. PKC activity is expressed as the difference between the activity assayed with and without Ca^{2+}, phosphatidylserine and diolein. Values are counts per min/mg protein. Protein was assayed by the Bradford method [16].

2.5. Western blot analysis

Samples of cytosolic and particulate fraction protein were separated by SDS-PAGE on 8% gels according to the method of Laemmli [17]. The separated proteins were electrophoretically transferred to a nitrocellulose membrane overnight. Non-specific binding sites were blocked by incubating the membrane with 5% fat-free dried milk in TBST (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20). Anti-PKC antibodies were added at the appropriate concentration and incubated for different times at room temperature. The membrane was washed with TBST and incubated with the secondary antibody. The blots were developed using ECL reagents, visualized on Kodak X-ray films and the immunoreactive bands were quantified by densitometric scanning (Studio Scann IISI, Agfa). Rat brain protein extracts were run in parallel as positive controls for the detection of PKC isoforms.

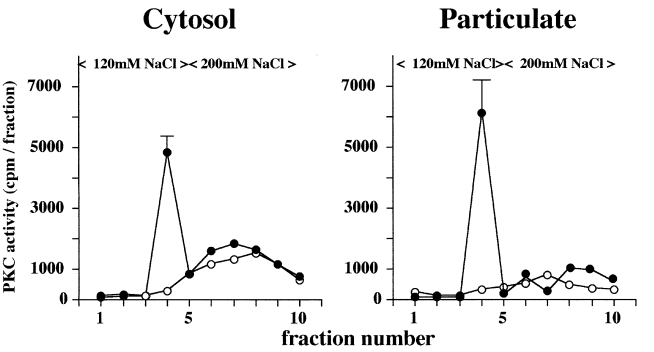

Fig. 1. Chromatography of cytosolic and particulate-associated protein kinase C from human placental stem villi vessels on a DEAE-cellulose column. PKC activity in the eluted fractions was assayed as described in Section 2 and is expressed cpm: (●) in the presence of Ca^{2+}, phosphatidylserine and diolein, (○) in the presence of EGTA, without phosphatidylserine or diolein. Results are representative of three experiments.

2.6. Data analysis

Student's unpaired *t*-test was used to determine statistical significance. *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Characterization and activation of protein kinase C

PKC activity was present in both the cytosolic and particulate fractions prepared from unstimulated stem villi vessels of human term placenta. DEAE-cellulose column chromatography (Fig. 1) showed a main peak of phospholipid- and Ca^{2+}-dependent activity eluted with buffer containing 120 mM NaCl. The second peak, eluted with 200 mM NaCl, was not regulated by Ca^{2+} or by phospholipid, and may have been the proteolytically generated catalytic domain of the enzyme (PKM). Unstimulated PKC activity was nearly equally distributed between the soluble (56 ± 8%) and particulate fractions (44 ± 7%) ($n = 6$).

Incubation with increasing concentrations of PMA, a potent phorbol ester, for 15 min, caused a gradual loss of PKC activity from the cytosol. The maximal effect (89 ± 4% loss) occurred with 1 μM PMA (Fig. 2A). This decrease was correlated with a parallel increase in particulate-bound PKC activity (up to 6-fold) (Fig. 2B). In contrast, the biologically inactive phorbol ester, 4αPDD, was without significant effect. Incubation of stem villi vessel samples with ET-1 resulted in a moderate but significant change in PKC activity from the cytosol towards the particulate fraction. ET-1 (1 μM) de-

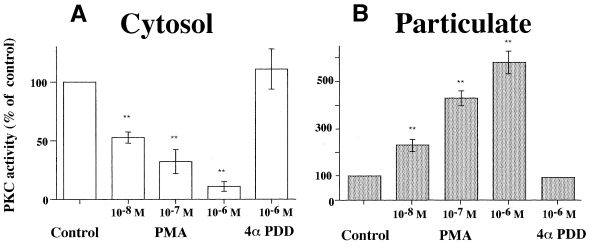

Fig. 2. Effects of PMA and 4αPDD on PKC activity in the cytosolic (A) and particulate fractions (B) of human placental stem villi vessels. PKC activity was determined as described in Section 2. Data are means ± S.E.M. for three series of experiments done in triplicate. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control value.

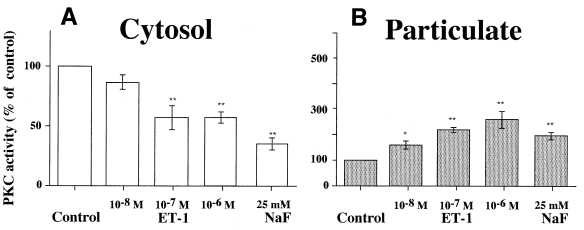

Fig. 3. Effects of NaF and ET-1 on PKC activity in the cytosolic (A) and particulate fractions (B) of human placental stem villi vessels. PKC activity was determined as described in Section 2. Data are means ± S.E.M. of three series of experiments done in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control value.

Table 1
Percent distribution of PKC isoforms in the cytosolic and particulate fractions of human placental stem villi vessels

Isoform	Distribution (%)	
	Cytosolic	Particulate
α	15 ± 2	85 ± 3
β_1	40 ± 4	60 ± 4
β_2	25 ± 3	75 ± 5
ε	35 ± 7	65 ± 6
ζ	40 ± 5	60 ± 8

Cytosol and particulate samples were analyzed by SDS-PAGE and immunoblotted using PKC-isoform specific antisera, as described in Section 2. Specific immunoreactive bands were quantified by densitometric scanning. The values are percent of total band intensity. Values are means ± S.E.M. of three experiments.

creased cytosolic PKC activity (43 ± 10%) and increased the particulate fraction activity (up to 3-fold). Incubation with 25 mM NaF, a direct activator of G-proteins, also changed the subcellular distribution of PKC activity: a 63 ± 10% decrease in the cytosolic fraction and a 2-fold increase in the particulate fraction (Fig. 3A,B).

3.2. Identification and intracellular distribution of PKC isoforms

Isozyme-specific antibodies were used to show the presence of at least five PKC isoforms in the cytosol and particulate fractions of the muscular layer of stem villi vessels in human term placenta. The main ones were PKCα, PKCβ_1, PKCβ_2, and PKCζ, with traces of PKCε (Fig. 4). Molecular weight markers were run in parallel and all the isoforms examined were compared to rat brain cytosolic extracts. The specificity of each immunoreactive band was shown by running the blots with the appropriate peptide antigens.

PKCα gave a single immunoreactive band at approximately 80 kDa. It was mainly in the particulate fraction, although there was a weak signal in the cytosolic extract. Several bands were recognized by PKCβ_1 antibody: two major bands with apparent molecular weights of 80 and 76 kDa and smaller minor immunoreactive bands. All these forms were present in both fractions and were not detected by PKCβ_1 antibody incubated with the corresponding antigenic peptide. Thus, the major bands may represent intact PKCβ_1 and the minor ones may be modified forms generated by proteolysis. Several forms of PKCβ_2 were also detected. There was a band at approximately 80 kDa, mainly in the particulate fraction, and additional bands of lower apparent molecular weights, all blocked by the peptide antigen. The isoforms PKCγ and

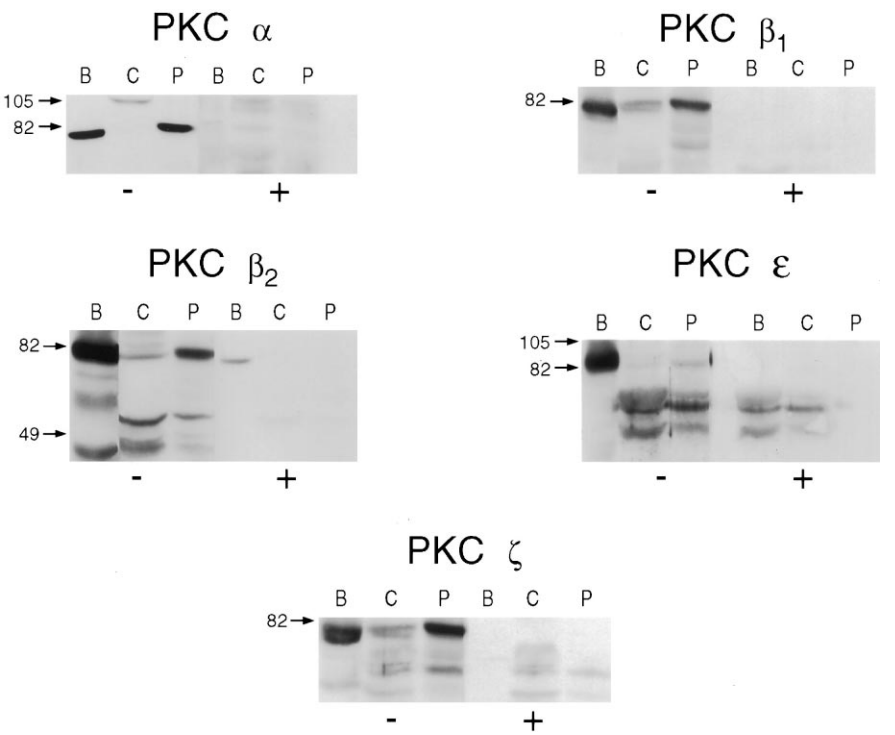

Fig. 4. Representative immunoblot analysis of PKC isoforms in the cytosol and particulate fractions isolated from human placental stem villi vessels. Samples of cytosolic (C) and particulate (P) fractions were prepared and analyzed as described in Section 2. Proteins (80 μg/lane) were separated on 8% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with isoform-specific anti-PKC antisera. The specificity of staining was confirmed by preincubating antisera with (+) and without (−) the appropriate peptide antigen. B: A rat brain cytosol fraction used as positive control. Arrows indicate the positions of the molecular weight markers (kDa).

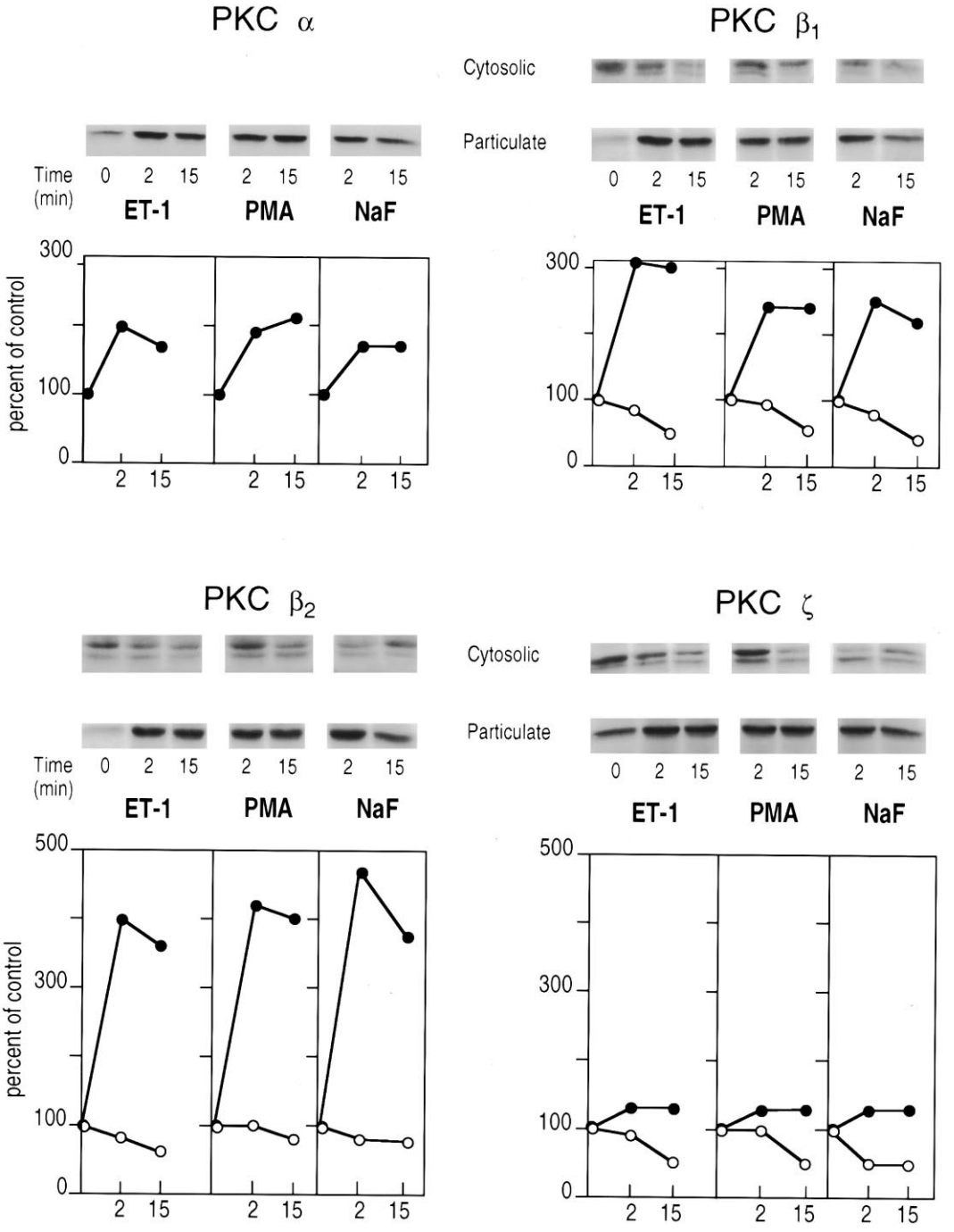

Fig. 5. Changes in distribution of immunoreactive PKCα, PKCβ$_1$, PKCβ$_2$, and PKCζ of human placental stem villi vessels stimulated for 2 and 15 min in the presence of either 1 μM ET-1, 1 μM PMA or 25 mM NaF. Proteins from the cytosolic (40 μg) and particulate (30 μg) fractions were analyzed as described in Fig. 4. The results shown are representative of two experiments. The upper panels show immunoblots of cytosolic and particulate fractions. The lower panels show the quantification by scanning densitometry of the immunoblots from cytosolic (○) and particulate fractions (●).

PKCδ were not detected in conditions which revealed their presence in a rat brain control, even when large amounts of protein (up to 120 μg) were run. PKCε was poorly represented, and was detected as a single specific band with an apparent molecular weight of 90 kDa in both fractions, mostly in the particulate fraction. In contrast, PKCζ appeared as a doublet with estimated molecular weights of 80–76 kDa. The other isoforms were not investigated.

The amounts of these isoforms were not quantified, since the antisera had different affinities for their respective antigen and thus for the corresponding PKC isoforms. However, densitometric analysis of the relative amounts of the cytosol and particulate forms for a given isoform were determined. Table 1 shows that all the isoforms were found mainly in the particulate fraction.

3.3. Subcellular redistribution of PKC isoforms

The subcellular redistribution of the four chiefly expressed

PKC isoforms in response to treatment with ET-1, PMA and NaF was examined by Western blot analysis (Fig. 5). All the PKC isoforms were translocated from the cytosolic to the particulate fractions upon stimulation. Treatment with either 1 μM ET-1, 1 μM PMA or 25 mM NaF for 15 min resulted in a time-dependent loss of PKCβ$_1$, PKCβ$_2$ and PKCζ in the cytosolic fractions, with a concomitant increase in particulate-associated immunoreactive PKCβ$_1$, and PKCβ$_2$. The decrease in PKCα in the soluble fraction was not analyzed because this isoform is weakly expressed in this fraction (15% of total PKCα). Although a decrease in cytosolic PKCζ was observed upon stimulation by these different stimuli (50% loss), no significant increases in particulate-associated immunoreactive PKCζ were detected. These stimuli were more effective in increasing particulate-associated PKCβ$_2$ (up to 4-fold above the control values) than PKCα and PKCβ$_1$ (2-fold and 3-fold above the control values).

4. Discussion

This study demonstrates the presence of protein kinase C in the muscular layer of stem villi vessels from human term placenta. Both cytosol and particulate PKC activities were eluted as single peaks from DEAE-cellulose. Under basal (unstimulated) conditions, PKC activity appears to be equally distributed between the soluble (cytosol) and particulate-associated forms. A similar high percentage of particulate-bound PKC activity has been reported in some metabolically active tissues such as brain, liver, and skeletal muscles, and may be a preactivated form of PKC.

It is generally agreed that activation of PKC is associated with its redistribution to several intracellular compartments where endogenous protein substrates are presumably located [1]. Therefore, the translocation of PKC activity from the cytosol to the particulate fraction is an index of enzyme stimulation [18]. PMA, a potent activator of this kinase, causes almost complete loss of PKC activity from the cytosolic fraction, and a marked increase in the particulate fraction. In contrast, the inactive phorbol ester 4αPDD was without effect, indicating that the translocation of PKC induced by PMA is associated with the activation of PKC. ET-1 produced a moderate but significant redistribution of PKC activity. It is well known that physiological agonists produce more transient and less intense signals than those induced by direct activators such as PMA. The positive effect of fluoride is evidence that PKC activation is mediated by the stimulation of G-protein(s). This complements and extends previous data showing that ET-1 [15] and fluoride (Mondon, personal communication) cause the accumulation of inositol phosphates in stem villi vessels, suggesting that ET-1 activates PKC via a G-protein(s)-coupled phosphoinositide breakdown.

Using Western blot we detected the presence of at least five PKC isoforms (PKCα, PKCβ$_1$, PKCβ$_2$, PKCε and PKCζ) in this vascular smooth muscle. PKCγ and PKCδ were not detected and other isoforms were not assayed. The apparent molecular weights of these proteins, determined by comparison with molecular weight markers, are close to the published values. The presence of each isoform was confirmed by comparison with the immunoreactive bands observed in rat brain cytosol extracts. The immunospecificity of the bands was also checked by performing the blots in the presence of the appropriate peptide antigen. The relative amount of each PKC isoform is difficult to determine, since the antibodies had different binding affinities. All the isoforms were present in both the cytosol and particulate fractions. Quantitation of immunoreactive bands revealed that all the PKC isoforms are mainly in the particulate fraction, and therefore presumably active. This result is in agreement with enzyme activity data showing a high content of particulate-bound PKC activity. Ruzycky et al. [19] recently reported that the majority of human placental syncytiotrophoblast PKC was bound to the plasma membranes.

PKCα, an ubiquitous isoform, appears to play a general role in cell physiology and seems to be involved in the regulation of rat coronary smooth muscle cell proliferation [20]. The two splice variants of PKCβ, PKCβ$_1$ and PKCβ$_2$, detected in stem villi vessels, have been identified in a variety of tissues in a variable ratio. PKCβ$_2$ became associated with actin filaments when activated [21], suggesting its involvement in the contractile process. It should be noted that feto-placental vessels contain a large amount of α-smooth muscle actin isoform [22]. The absence of PKCγ was expected, since this isoform is mainly found in the brain or brain-derived tissues. Nevertheless, it has been found in some cell lines [23] and in the human syncytiotrophoblast [19]. We found no evidence for PKCδ, although this isoform appears to be abundant in several vascular smooth muscles [24], and PKCε was poorly detected. This latter isoform seems to be involved in Ca^{2+}-independent contraction in the ferret aorta [25]. In contrast, the immunoreactivity of the atypical isoform PKCζ was intense, suggesting that this isoform is present in high amounts. This isoform was detected as a specific doublet of immunoreactive bands, as in several other tissues and cell types. They may represent different phosphorylated states of the isoform, though it is possible that one band may be due to cross-reaction with conventional PKC isoforms [26]. This widely distributed isoform appears to play a significant role in the mitogenic pathway [27] by linking membrane signalling to nuclear events.

The existence of PKC isoforms from three distinct groups and the presence of both Ca^{2+}-dependent and Ca^{2+}-independent isoforms indicate that PKC plays a complex, multifunctional regulatory role in the muscular layer of human placental stem villi vessels. The presence of Ca^{2+}-independent isoforms of PKC, as in other vascular smooth muscle, strongly suggests that these isoforms mediate a phosphatidyl choline hydrolysis-coupled mechanism which generates DAG without any concomitant mobilization of intracellular Ca^{2+}. This phospholipid degradation has been shown to be involved in proliferation and differentiation processes.

This pattern of PKC isoforms is consistent with that previously reported for other vascular smooth muscle cells [2]. PKCα and PKCβ are the primary PKC isoforms in rabbit and rat aorta and in swine carotid artery [28,29]; PKCε and PKCζ have been found in rat and ferret aorta smooth muscle cells [25], whereas PKCδ was not detected [30]. PKCα, PKCδ, and PKCζ have also been identified in the human renal artery [25].

The intracellular redistribution of individual PKC isoforms in response to different stimuli was then examined. All four mainly expressed PKC isoforms were translocated from the cytosolic to the particulate fractions upon stimulation with either ET-1, PMA or NaF. Apparently there was no differential activation of the various PKC isoforms examined. None

of the agonists tested led to increases in particulate-associated PKCζ immunoreactivity. A possible translocation of this isoform to the nucleus and perinuclear space, as observed in a variety of cell types including aortic smooth muscles [30], cannot be excluded. Complex patterns of PKC translocation have been reported in various tissues and may explain the diversity of functions modulated by this kinase [31].

The present results show that the increase in particulate-associated PKC immunoreactivity was associated with enhanced Ca^{2+}-dependent PKC activity reflecting PKC activation. The regulatory role of PKC in vascular smooth muscle functions ranging from short-term effects (contraction) to long-term events (cell growth, proliferation, differentiation) has been demonstrated [1]. This study suggests that in stem villi vessels, as in other vascular smooth muscles [32–35], PKC may play a role in mediating ET-1 responses.

In conclusion, our data demonstrate for the first time the presence of several PKC isoforms in human placental stem villi vessels. Stimulation by different stimuli induced redistribution of PKC activity from the cytosolic to the particulate fractions associated with subcellular redistribution of individual PKC isoforms. The translocation of PKC by ET-1 suggests a potential role of this enzyme in the endothelin-stimulated signalling pathway of the placental stem villi vessels.

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