Contribution of protein kinase C to ET-1-induced proliferation in human myometrial cells

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Tertrin-Clary, C., I. Eude, T. Fournier, B. Paris, M. Breuiller-Fouché, and F. Ferre. Contribution of protein kinase C to ET-1-induced proliferation in human myometrial cells. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E503–E511, 1999.—The role of protein kinase C (PKC) in endothelin-1 (ET-1)-induced proliferation of human myometrial cells was investigated. ET-1 dose dependently stimulated DNA synthesis and the number of cultured myometrial cells. Inhibition of PKC by calphostin C or Ro-31-8220 or downregulation of PKC eliminated the proliferative effects of ET-1. The failure of two protein tyrosine kinase (PTK) inhibitors (tyrphostin 51 and tyrphostin 23) to affect ET-1-induced proliferation supports the hypothesis of noninvolvement of the tyrosine kinase signaling pathway in this process. The expression and distribution of PKC isoforms were examined by Western blot analysis. The five PKC isoforms (PKC-α, -β1, -β2, -γ, -ε) evidenced in human myometrial tissue were found to be differentially expressed in myometrial cells, with a predominant expression of PKC-α and PKC-γ. Treatment with phorbol 12,13-dibutyrate (PDBu) resulted in the translocation of all five isoforms to the particulate fraction, whereas ET-1 induced a selective increase in particulate PKC-β1, PKC-β2, and PKC-ε. Our findings that multiple PKC isoforms are differentially responsive to ET-1 or PDBu suggest that they play distinct roles in the myometrial growth process.

endothelin; myometrium

ENDOTHELIN-1 (ET-1), originally isolated from endothelial cells, is a 21-amino acid peptide with potent vasoactive properties. Further studies have revealed the existence of additional isopeptides, ET-2 and ET-3, which differ from ET-1 by two and six amino acids, respectively. The three isopeptides are highly homologous and bind to distinct receptors, designated ET1 and ET2 (see Ref. 35 for review). Human uterine smooth muscle cells express the ET1 receptor subtype, which is ET-1 selective (20, 25). This vasoactive peptide synthesized in intrauterine tissue (33) can affect myometrial cells in a paracrine fashion to induce contractility. In humans, ET-1-induced uterine contractions occur via the selective activation of ET1 receptors (3, 21, 31), which are functionally linked to phospholipase C and protein kinase C (PKC) (7, 8). The discovery that ET-1 may also regulate proliferation of cultured human myometrial cells (6) provides new perspectives when the biological activity of ET-1 is considered. This hypothesis may be consistent with the presence of ET1 receptor.
cultured human myometrial cells and studied their regulation by a phorbol ester, phorbol 12,13-dibutyrate (PDBu), and ET-1. Another objective was to define, among the isoforms of PKC evidenced in cultured myometrial cells, those that are required for ET-1-induced cell proliferation.

**MATERIALS AND METHODS**

Chemicals. Dulbecco's modified Eagle's medium (DMEM) with or without phenol red and fetal calf serum (FCS) was from Gibco Life Technologies (Cergy-Pontoise, France). [6-3H]thymidine (25 Ci/mmol), Hybond-C membranes, the enhanced chemiluminescence detection system (ECL), and X-ray films were obtained from Amersham International (Chalfont, Buckinghamshire, UK). ET-1 was from Neosystem (Strasbourg, France). Antibodies against PKC-α, PKC-δ, PKC-ε, and PKC-ζ were from Gibco Life Technologies. Those were raised in rabbits against peptides 313–326 from PKC-α, 662–673 from PKC-δ, 726–737 from PKC-ε, and 577–592 from PKC-ζ. Antibodies against PKC-β1 and PKC-β2 were from Santa Cruz Biotechnology (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 International, and prestained molecular-weight markers were from Bio-Rad. Epidermal growth factor (EGF) was purchased from Chemicon International (Temecula, CA). The PKC inhibitor Ro-31-8220 was a generous gift of Dr. Bradshaw (Roche Discovery, Welwyn, Hertfordshire, UK). Phorbol 12,13-dibutyrate (PDBu), leupeptin, Nonidet P-40, phenylmethylsulfonyl fluoride (PMSF), and other drugs and chemicals were used of the highest quality available from Sigma (St. Louis, MO).

Preparation and culture of myometrial cells. Human myometrium was obtained from eight women (34–46 yr old) undergoing hysterectomy for benign gynecological indications. None of the patients was under hormonal treatment at the time of surgery. Tissue samples were excised in the uterine corpus from normal muscle (myometrial outer layer) in areas free of macroscopically visible anomalies. This study was approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (Paris-Cochin, France).

Human myometrial cells were harvested as previously described (10) from muscle explants and were cultivated in 20% FCS in DMEM supplemented with antibiotics. When cells could be observed growing out from the explants, the culture medium was replaced by 10% FCS in DMEM. Cells were confluent 21 days after tissue collection and then were subcultured every 7 days by trypsinization. For selected cell growth experiments, myometrial cells were obtained between passages 3 and 6, with no noticeable difference in results observed with cells from individual passages or with cells obtained from different uteri at either the proliferative or the secretory phase of the menstrual cycle. Confluent myometrial cells were identified by their positive reaction with monoclonal antibodies against smooth muscle α-actin, smooth muscle-1 (SMA) and SM2 myosin heavy chains, and desmin and by the typical “hill and valley” microscopic findings. Each population of myometrial cells studied came from a separate patient.

Assessment of proliferation. Myometrial cells (50,000 cells/well) were cultured to subconfluence in 24-well dishes in the presence of 10% FCS for 24 h. The cells in exponential growth were then transferred to serum-deprived media for 72 h to achieve quiescence. Quiescent cells were incubated for 48 h as previously described (6) in serum-free media in the presence of various concentrations of ET-1 or EGF dissolved in sterile water. In some experiments, PDBu and inhibitors of PKC and PTk were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration was set in all cases at the maximal value of 0.1% and did not affect cellular growth. In combination experiments, protein kinase inhibitors were added 30 min before incubation with ET-1 or EGF. Serum-free DMEM with 0.1% DMSO was used as a negative control and DMEM with 10% FCS as a positive control. [3H]thymidine (0.4 µCi/well) was added during the final 24 h of incubation. After incubation, cells were washed twice with phosphate-buffered saline (PBS) without Mg2+ and Ca2+, fixed with 5% trichloroacetic acid, washed twice with 100% ethanol, and solubilized with 0.5 N sodium hydroxide. Cell-associated radioactivity was measured by scintillation counting. All experiments were performed in quadruplicate.

Cell numbers were determined in separate experiments. Briefly, quiescent human myometrial cells were incubated with similar treatments. After 72 h, cells were trypsinized and counted with a hemocytometer. The viability of cells was assayed by trypan blue exclusion. Six replicate wells were used for each test condition.

Cell stimulation and extraction of PKC. Human myometrial cells (106 cells/75-cm2 flask) were cultured to subconfluence in the presence of 10% FCS for 48 h. Only cells at passage 4 were used here. Subconfluent cells maintained for 3 days in serum-free media were washed twice with PBS and incubated in culture medium containing 100 nM PDBu or 100 nM ET-1 for the indicated times at 37°C in a humidified atmosphere of 95% air-5% CO2. Incubation was stopped by aspiration of the medium, which was followed by two washes with cold PBS. The cells were then scraped into 20 mM Tris·HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM EGTA, 2 mM EDTA, 50 mM mercaptoethanol, 2 mM PMSF, 5% glycerol, and 40 µg/ml leupeptin and were sonicated twice for 10 s. The homogenates were centrifuged for 15 min at 1,000 g to remove cell debris and nuclei. After ultracentrifugation for 60 min at 100,000 g, the resulting supernatant was designated as the cytosolic fraction. The pellet was resuspended in the same buffer containing 1% Nonidet P-40 and was gently mixed for 45 min at 4°C. After centrifugation for 30 min at 100,000 g, the supernatant was saved and constituted the particulate fraction.

Protein contents were estimated with the Bradford protein assay (5).

In comparison studies, the same myometria were used for preparation of both crude homogenates and cell cultures. The PKC redistribution in human myometrial tissue was performed as previously reported (8).

Western blot analysis. Equal amounts of proteins (40 µg) from cytosolic and particulate fractions were separated by SDS-PAGE on 8% gels according to the method of Laemmli (23). The separated proteins were electrophoretically transferred to a nitrocellulose membrane overnight as previously described (39). Nonspecific 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 with ECL reagents and 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.

Statistical analysis. Data are presented as means ± SE. The statistical significance of differences in the results was evaluated by one-way ANOVA and Scheffé’s multiple comparison test. Significance was set at $P < 0.05$.

RESULTS

Effect of ET-1 on DNA synthesis and cell growth in human myometrial cells. We had previously shown that ET-1 stimulates the incorporation of $[^3H]$thymidine into DNA in human myometrial cells cultured in the presence of 0.5% FCS and increases the cell number (6). When added to quiescent cells cultured in serum-free conditions, ET-1 exhibited a dose-dependent DNA synthesis increase, with maximal stimulation (170% of the serum-free control) occurring at 100 nM ET-1 (Fig. 1A). The dose-response analysis gave an EC$_{50}$ value of 11 nM. The concomitant increase in cell counts reached statistical significance only at 100 nM ET-1, with maximal stimulation of 168% of the serum-free control (Fig. 1B). The increase in $[^3H]$thymidine incorporation and cell number induced by 100 nM ET-1 was less than that for 10% FCS.

Effect of PKC and PTK inhibition on ET-1-induced proliferation. To assess the contribution of PKC to ET-1-stimulated DNA synthesis in myometrial cells, we examined the effect of calphostin C and Ro-31-8220. Figure 2 shows that both compounds inhibited, in a dose-dependent manner, DNA synthesis induced by 100 nM ET-1 without affecting basal $[^3H]$thymidine incorporation. The maximal inhibitory effect obtained with 1 nM of PKC inhibitors was ~80% of ET-1 stimulation. Similarly, pretreatment of myometrial cells with 1 nM calphostin C or 1 nM Ro-31-8220 significantly decreased the cell number induced by 100 nM ET-1 stimulation and evoked 80% inhibition (data not shown). In contrast, the increase in DNA synthesis in response to 100 nM ET-1 was not significantly affected by pretreatment with tyrosine kinase inhibitors (tyrphostins 23 and 51) at any concentration tested (0.01–1 mM). None of the inhibitors tested was toxic to human myometrial cells under our conditions as assessed by trypan blue exclusion.

Prolonged treatment with a phorbol ester is known to induce the depletion of PKC. This was confirmed in myometrial cells by immunoblot analysis of the conventional PKC isoforms (see Fig. 7). As shown in Fig. 3, when myometrial cells were treated for 36 h in the presence of 100 nM PDBu, ET-1 did not increase the cell number. Under the same conditions, ET-1 had no significant effect on $[^3H]$thymidine incorporation (data not shown).

Effects of PKC or PTK inhibition on EGF-induced $[^3H]$thymidine incorporation. Previous investigations demonstrated that EGF (1–20 nM) stimulated $[^3H]$thymidine incorporation in a concentration-dependent manner.
PKC isoforms in cultured myometrial cells. Three conventional PKC isoforms were abundantly expressed, whereas PKC-β2 and PKC-ε were poorly represented.

A comparative study with results obtained with myometrial tissue was achieved. No differences in PKC isoform expression were detected between tissue and cultured cells. Scanning densitometric quantification of the Western blot PKC immunobands visualized in Fig. 5A showed that the amounts of total immunoreactive PKC-α and PKC-ζ were 2.5-fold and 2.2-fold greater, respectively, in cells than in tissue. In contrast, the quantities of the other PKC isoforms, PKC-β2, PKC-ζ, and PKC-ε, did not appear to be very different (136, 70, and 65%, respectively, compared with tissue).

Comparative subcellular distribution of the various PKC isoforms in myometrial cells and in myometrial tissue revealed some differences. Whereas PKC-α, PKC-β2, and PKC-ζ were equally distributed in myometrial cells and myometrial tissue, the other two PKC isoforms, PKC-β2 and PKC-ε, were found to be present solely in the particulate fraction (Fig. 5B). It should be noted that PKC-β2 exhibited several lower-molecular-weight species despite the presence of various anti-proteases and that PKC-ε appeared to be poorly represented in both myometrial tissue and myometrial cells.

Subcellular redistribution of PKC isoforms. The effects of PDBu and ET-1 treatments on the relative levels of various PKC isoforms in subconfluent serum-deprived human myometrial cells are shown in Fig. 6.

Acute treatment (2 and 20 min) with 100 nM PDBu induced an increase in particulate-associated PKC immunoreactivity of all isoforms tested in a time-dependent manner. Marked and sustained increases in particulate immunoreactivity of PKC-α, PKC-β2, and PKC-ζ were observed (330, 390, and 270% of the control level, respectively), whereas a slight and more transient enhancement of particulate immunoreactive PKC-β2 and PKC-ε was detected (150 and 250%, respectively). Interestingly, upon PDBu stimulation, PKC-ζ was resolved as two bands. An additional band of higher molecular weight appeared.

Incubation of myometrial cells in the presence of 100 nM ET-1 elicited a different pattern in intracellular PKC redistribution. Whereas a modest, transient change in immunoreactive particulates PKC-β2 and PKC-β2 was seen (150 and 170% of the control level), a more pronounced and sustained increase in particulate

The maximum effect obtained at 15 nM EGF was ~280% of unstimulated serum-free control. [3H]thymidine incorporation in response to 15 nM EGF was weakly blocked by 1 nM calphostin C, whereas 1 nM Ro-31-8220 had no effect (Fig. 4). Depletion of PKC by a 36-h treatment with 100 nM PDBu did not prevent an increase in EGF-induced DNA synthesis. By contrast, tyrphostin 51, which is a potent inhibitor of the EGF receptor (16), reduced by ~58% the increase in [3H]thymidine incorporation by 15 nM of EGF.

Expression and intracellular distribution of PKC isoforms in human myometrial cells. Western blot analysis with isoform-specific antibodies revealed the presence of at least five PKC isoforms in cultured myometrial cells. Three conventional PKC isoforms (PKC-α, PKC-β1, and PKC-β2), one novel PKC isoform (PKC-ε) in small amounts, and one atypical PKC isoform (PKC-ζ) were specifically detected. PKC-γ and PKC-δ were not identified in our conditions, and the presence of other isoforms was not investigated. The specificity of the immunobands was tested by competition in the presence of the appropriate peptides used as immunogens. Figure 5A shows a representative Western blot analysis of PKC isoenzyme expression in human myometrial cells.

Scanning densitometric analysis of the immunobands showed that PKC-α, PKC-β1, and PKC-ζ were nearly equally distributed between the soluble (53.4 ± 2.9, 47 ± 3, and 59 ± 4%) and particulate fractions (46.6 ± 2.9, 53 ± 3, and 41 ± 4%, respectively; n = 3). In contrast, PKC-β2 and PKC-ε appeared to be entirely particulate. PKC-α, PKC-β1, and PKC-ζ were abundantly expressed, whereas PKC-β2 and PKC-ε were poorly represented.
Fig. 5. A: representative immunoblot analysis of expression of PKC isoforms in cytosol and particulate fractions isolated from human myometrial cells (subculture 4) compared with human myometrial tissue. Samples of cytosolic (C) and particulate (P) fractions were prepared and analyzed as described in MATERIALS AND METHODS. Proteins (40 µg/lane) were separated on 8% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with isoform-specific anti-PKC antibodies. Rat brain extract (B) was used as positive control. Arrows indicate positions of molecular mass marker.

B: quantitation of scanning densitometric analysis of immunoreactive bands. Distribution of PKC isoforms in particulate (P) and cytosolic (CY) fractions of human myometrial tissue (T) compared with human myometrial cells (C). Values are percentages of total band intensity ± SE for 3 separate experiments with 3 different patients.
PKC-ε (270%) was observed. However, ET-1 had no detectable effect on the increase in immunoreactive PKC-α and PKC-ζ in the particulate fraction.

Long-term effect of PDBu on total PKC isoform levels. To further characterize the role of PKC in the mitogenic action of ET-1, we studied the effect of prolonged treatment with PDBu on the PKC content of the cells. Downregulation of PKC induced by prolonged exposure of myometrial cells to PDBu is shown in Fig. 7. Long-term (36 h) incubation with 2 µM PDBu induced a decrease in the total immunoreactive amounts of the main PKC isoforms detected. PKC-α and PKC-β₁ were the most sensitive, as 55 and 70% were lost, respectively, after this treatment. Under the same conditions, PKC-β₂ and PKC-ζ immunoreactivities were decreased by only 10%.
Indeed, ET-1-induced [3H]thymidine incorporation was not significantly affected by tyrphostin 23 (a broad-range PTK inhibitor) or tyrphostin 51 (a potent inhibitor of the EGF receptor).

Next, we examined which PKC or PTK pathway was responsible for EGF-induced proliferation in myometrial cells. The expression of EGF receptors in human myometrial cells has been shown to regulate proliferative and antiproliferative effects in smooth muscle cells (32). The expression of this ubiquitous isoform was modified during the differentiation process and is correlated with the regulation of smooth muscle cell proliferation and cell cycle progression (40). The novel PKC-ε isoform has been shown to induce cell growth and proliferation in vascular smooth muscle (18), and PKC-β2 seems to be associated with actin microfilaments, suggesting a role in modulation of uterine contractility (17). In various cell systems, the atypical PKC-ζ isoform appears to mediate nuclear responses such as maturation, gene transcription, and proliferation.

In this study, we determined the activation of the various PKC isoforms identified in human myometrial cells by measuring the increase in their particulate fraction upon treatment by PDBu and ET-1. PDBu caused a marked and sustained translocation of all the PKC isoforms detected in these cells. However, as often
reported, the magnitude and time course of the particulate-associated PKC isoforms showed some differences according to the isoform considered. Although PKC-ζ contains a single-system domain, its redistribution after exposure to PDBu may be observed. No information is yet available as to whether its translocation resulted from cross-reaction between conventional PKCs and anti-PKC-ζ antibody (1) or was the consequence of the high dose of phorbol ester used. Upon PDBu stimulation, we noted that PKC-ζ appeared to be present as a doublet, probably reflecting changes in the phosphorylation state of the isoform as a consequence of its activation (30). Under the same conditions, ET-1 can also induce rapid and selective activation of PKC. As observed for phorbol ester, differential sensitivity of the different PKC isoforms was noted. Whereas ET-1 induced an increase in immunoreactive PKC-β1, PKC-β2, and PKC-ε in the particulate fraction, no variations in PKC-α and PKC-ζ were observed. At all times tested, the ET-1 responses were of lower magnitude than those induced by the direct PKC activator, as reported in most cell systems.

The inability to detect an ET-1-induced increase in particulate PKC-α and PKC-ζ may be explained by a redistribution of the two PKC isoforms into other intracellular sites, such as the perinuclear area or within the nucleus as reported in several types of smooth muscle cells, which suggests intranuclear functions and transcriptional control (12, 28, 32, 41, 42). Such a failure to detect translocation of PKC-α and PKC-ζ in response to agonists has also been previously observed for cardiomyocytes (11, 32). It may be considered that individual isoforms of PKC should be associated with a particular cellular structure that contains the specific protein substrates for these isoforms.

Downregulation of PKC by prolonged treatment with PDBu abolished the mitogenic action of ET-1 in myometrial cells. This observation is in agreement with previous data that report inhibition of growth in various cell types after depletion of PKC content (15, 37) and confirms the role of PKC in the mitogenic properties of ET-1. Western blot analysis revealed a marked decrease in the total amount of PKC-α and PKC-β1 suggesting that these two PKC isoforms may be involved in the regulation of ET-1-induced mitogenesis. However, we cannot exclude a role for the other isoforms in this process. The failure to observe a consequent loss of PKC-β2 and PKC-ζ during this treatment may be explained by differential phorbol ester depletion of PKC isoforms. More prolonged treatment in the presence of PDBu might be necessary to induce complete downregulation of all PKC isoforms. It should be pointed out that the mechanisms through which ET-1 modulates cellular growth involve activation of multiple signal transduction pathways. In addition to stimulating phosphoinositide hydrolysis through phospholipase C activation (7), ET-1 is able to stimulate phospholipase D (29) in the myometrium. We cannot rule out that both pathways may also contribute to the proliferative action observed in our cellular model.

These data suggest that ET-1-induced proliferation in human myometrial cells is mediated by a PKC-dependent pathway. The differential changes observed in the levels of PKC isoforms indicate that individual PKC isoforms may elicit specific cell responses. In relation to this problem, overexpression of PKC isoforms in various cell lines has been reported, and a correlation with the development of the malignant phenotype was observed (27). PKC activation appears to be involved in the smooth muscle cell-extracellular matrix interaction (18), suggesting a role for this enzymatic system in the metastatic process (24). Moreover, the PKC isoenzyme expression pattern has been associated with proliferative activity in endometrial tumor cells (4). Further studies are required to determine the role of each PKC isoform in human uterine smooth muscle in normal and pathological processes. In particular, the use of antisense oligonucleotides specific to an isoform may help to elucidate the role of each individual PKC isoform.

In summary, the results of this study indicate that PKC may regulate ET-1-induced proliferation in human myometrial cells. We also found that ET-1 caused selective intracellular redistribution of PKC isoforms, providing evidence that they regulate different functions. The main question that remains unanswered is whether PKC may regulate ET-1-induced proliferation in human myometrium during pregnancy and in uterine disorders such as leiomyomas.

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