Contribution of PKC-dependent and -independent processes in temporal ERK regulation by ET-1, PDGF, and EGF in rat myometrial cells

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The smooth muscle tissue of the uterus, the myometrium, undergoes diverse physiological changes such as hypertrophy, hyperplasia, contraction, and apoptosis, which are critical for uterine activities. These biological functions are modulated by multiple hormonal factors including steroid and peptide hormones, growth factors, prostaglandins, and neurotransmitters.

Endothelin-1 (ET-1), a peptide hormone, as well as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), have long been described as mitogens in many cell types including myometrial cells (2, 10, 18, 32). In rat myometrial cells, we recently showed that ET-1 and PDGF stimulate DNA synthesis in an extracellular signal-regulated kinase (ERK)-dependent manner. Activation of ERK by ET-1 occurs through the sequential activation of PKC, Src, and Ras, which leads to ERK activation. PKC is a family of serine/threonine kinases that is subdivided into three groups on the basis of structural and biochemical properties: the conventional (α, β1, β2, and γ), novel (δ, ε, η, θ, μ, and ν), and atypical (ζ and η/α) isoforms. Conventional and novel (c/n)PKC isoforms are activated by diacylglycerol (DAG), a product of phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis, and by phospholipid esters. In addition to DAG, cPKC requires Ca²⁺ for full activation (33). In contrast to c/nPKC, atypical (a)PKC are insensitive to DAG and are activated by phosphorylation. PKCζ is phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1), which is activated by phosphatidylinositol 3,4,5-trisphosphate (PIP₃), a product of the phosphoinositide 3-kinase (PI 3-kinase) activity (19). Our data strongly suggested that both c/nPKC and aPKCζ, which are activated downstream of phospholipase C (PLC) and PI 3-kinase, respectively, equally contribute to ET-1-induced ERK activation (32). By contrast, the mechanisms by which PDGF and EGF stimulate ERK activation in rat myometrial cells have not been studied. EGF and PDGF receptors belong to the family of receptor tyrosine kinases (RTK), which dimerize and autophosphorylate on tyrosine residues upon ligand binding. These two receptors, once phosphorylated, are known to recruit adaptor protein Grb2, which is associated with Sos, the specific exchange factor of Ras. This recruitment can occur directly for the PDGF receptor or indirectly through adaptor protein She for the EGF receptor. Recruited Sos then catalyzes the conversion of inactive Ras-GDP into active Ras-GTP, which then interacts with Raf-1. This kinase activates MEK (MAP kinase/ERK kinase), which in turn activates ERK. EGF and PDGF have also been shown to stimulate phosphoinositide turnover by recruiting and phosphorylating PLCγ1. This leads to the generation of DAG and mobilization of Ca²⁺ that may activate PKC (33).

PKC have also been described as a major MAP kinase pathway activator, but the mechanisms involved are multiple and seem to be, to some extent, cell-type specific. PKC can activate Raf-1 (17) or MEK (26, 41) through a direct phosphorylation. However, PKC may also stimulate Ras through the activation of protein tyrosine kinases such as Src family kinases (1, 14) or transactivation of a RTK such as EGF or PDGF receptor (16, 21, 35). In rat myometrial cells, ET-1 transactivates neither EGF nor PDGF receptors. In contrast, the ET-1-induced ERK activation is fully dependent on Src activity (32).

The purpose of this study was to analyze and compare the signal transduction mechanisms involved in the activation of ERK and the associated mitogenic effect by ET-1, PDGF, and EGF.

MATERIALS AND METHODS

Materials. ET-1 was from NeoSystem (Strasbourg, France), and PDGF-BB was from Peprotech (Tebu, Le Perray-en-Yvelines, France). PDGF-BB was from Peprotech (Tebu, Le Perray-en-Yvelines, France).
Frances). EGF, β-estradiol-3-benzoate, leupeptin, aprotinin, LiCl, phorbol 12-myristate 13-acetate (PMA), and phorbol 12,13-dibutyrate (PDBu) were from Sigma (St. Louis, MO). U-73122 and U-73343 were from Biomol (Tebu). Western blotting detection reagents were obtained from PerkinElmer (Boston, MA). Myo-[2-3H]inositol (10–20 Ci/mmol) and glutathione-Sepharose CL–4B were obtained from Amersham Pharmacia Biotechnology (Les Ulis, France). Ro-31-8220, Go-6976, and rottlerin were from Calbiochem (Meudon, France). Collagenase was from Boehringer Mannheim (Meylan, France). Poly- 
clonal antibody to ERK1/2 was from Zymed Laboratories (San Francisco, CA), and polyclonal anti-active ERK1/2 antibody was from Promega (Madison, WI). Monoclonal anti-pan-Ras antibody was from Upstate Biotechnology (Lake Placid, NY). Poly- 
clonal antibodies to PKC isoforms, culture media, and fetal calf serum were from Invitrogen (Cergy Pontoise, France). Horseradish peroxidase-conju-
gated anti-rabbit antibodies were from Dako (Trappes, France). All other reagents were of the highest commercially available grade.

Animals. Prepubertal Wistar female rats from Janvier (Le Genest Saint Isle, France), 21 days old, were housed for 7 days before use in an environmentally controlled room. Chow and water were available ad libitum. Rats were treated with 30 μg of estradiol for the last 2 days and were killed at 28 days of age by 1 min of carbon dioxide inhalation. All treatments were performed in accordance with the procedures outlined in the European guidelines for the care and use of experimental animals.

Myometrial cell preparation and culture. Primary cultures of myo-
metrial cells were prepared by collagenase digestion as previously described (2). The myometrial cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO2-95% humidified air. The medium was changed every 2 days, and cells were kept in serum-free medium for 24 h before experiments.

Measurement of [3H]inositol phosphates. Confluent myometrial cells seeded in 24-well plates were labeled by incubation for 48 h in serum-free medium supplemented with 5 μCi/ml myo-[2-3H]inositol (final concentration 10 μM). Cells were washed twice with Hanks’ balanced salt solution containing 20 mM HEPES (pH 7.5) and incubated at 37°C in fresh buffer with 10 mM LiCl. After 10 min, the agents to be tested were added at the indicated concentration, and incubation was further continued for the time indicated for the specific experiment. Reactions were stopped by aspiration of the incubation medium, followed by the addition of 1 ml of cold trichloroacetic acid (TCA, 7% wt/vol). Cells were detached by scraping on ice and centrifuged at 10,000 g for 15 min at 4°C. Total inositol phosphates (InsPs) were quantified as previously described (2). Results were expressed as counts per minute per well.

Analysis of phosphorylated ERK1/2. Serum-starved confluent myo-
metrial cells seeded in six-well plates were rinsed twice with Hanks’ balanced salt solution containing 20 mM HEPES (pH 7.5) and incubated in 2 ml of fresh medium for 10 min. Cells were then exposed to the agents tested. Reactions were stopped by aspiration of the incubation medium, followed by addition of 100 μl of cold solubilization buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM NaF, 10% glycerol, 10 mM Na4 P2O7, 200 μM Na3VO4, 10 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). Cells were detached by scraping on ice and centrifuged at 10,000 g for 20 min at 4°C. Detergent-extracted proteins (40 μg) were heated for 10 min at 95°C with Laemmli’s sample buffer and analyzed by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose sheets and were probed with polyclonal anti-active ERK1/2 antibodies (1:5,000). The blot was then stripped in glycine (0.1 M)-HCl (pH 2.2) and reprobed with polyclonal anti-ERK1/2 antibodies (1:5,000). The immunoreactive bands were visualized by an enhanced chemiluminescence system after incubation with horseradish peroxidase-conjugated swine anti-
rabbit IgG. Quantification of the developed blots was performed with a densitometer (Molecular Dynamics, Sunnyvale, CA).

Preparation of rat brain extract. One rat brain was washed with cold PBS and homogenized with an Ultra Turrax homogenizer in 5 ml of cold 50 mM Tris buffer, pH 7.4, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The homogenate was centrifuged for 15 min at 1,000 g at 4°C, and the pellet was discarded. The supernatant was used as positive control for PKC antibodies.

Western blot analysis of PKC isoforms. Detergent-extracted myo-
metrial cell proteins (50 μg) and proteins from the rat brain extract (10 μg) were analyzed by 7.5% SDS-PAGE and transferred on nitrocel-
lulose sheets. PKC isoforms were immunodetected with polyclonal isoform-specific antibodies (anti PKCα, β and ε: 1:1,000; anti PKCB and γ-δ: 1:500; anti PKCζ: 1:2,000). The immunoreactive bands were visualized by an enhanced chemiluminescence system after incubation with horseradish peroxidase-conjugated anti-rabbit IgG.

[3H]thymidine incorporation. Serum-starved myometrial cells (50% confluent) in 24-well dishes were incubated for 24 h with the various agents to be tested before [3H]thymidine (2 μCi/ml) was added to each well. Cells were incubated for an additional 24 h, and then reactions were terminated by aspiration of the incubation medium and addition of 0.5 ml of cold TCA (10% wt/vol). Radioactivity incorporated into TCA-precipitable material was recovered with 0.5 ml NaOH (1 N) and quantified by liquid scintillation counting.

Ras activation assay. Ras activation was determined by the Ras-
GTP pull-down assay with GST-RBD (amino acids 51–113 of human Raf-1) as described previously (32). This assay is based on the capacity of activated Ras to bind to the Ras-binding domain (RBD) of c-Raf-1. This domain, produced as a glutathione-S-transferase (GST) fusion protein, allows the selective precipitation of activated Ras in the presence of glutathione-Sepharose beads. To prepare the GST-
RBD fusion protein, Escherichia coli BL21 cells were transformed with pGEX-RBD plasmid. Bacteria from a 250-ml overnight culture were pelleted (2,500 g, 15 min, 4°C) and resuspended in 15 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cells were lysed by sonication, and NP-40 was added (0.5% final concentration). The lysate was cleared by centrifugation at 10,000 g for 15 min at 4°C, and the supernatant was incubated for 1 h in the presence of glutathione-Sepharose beads to recover the GST-RBD protein. Beads were then washed five times in the same buffer.

To analyze Ras activation, serum-starved confluent myometrial cells seeded in 50-cm2 plates were rinsed twice with Hanks’ balanced salt solution containing 20 mM HEPES, pH 7.5, and incubated in 4 ml of fresh medium for 10 min. Cells were then exposed to the agents to be tested. Reactions were stopped by aspiration of the medium and addition of 1 ml of cold Ras assay (RA) buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM NaF, 10% glycerol, 10 mM Na2P2O7, 200 mM Na3VO4, 10 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) and centrifuged at 10,000 g for 5 min at 4°C. The supernatants were collected and incubated in the presence of 8 μg of GST-RBD bound to glutathione-Sepharose beads for 1 h at 4°C in a 2-ml final volume to allow activated Ras to bind to GST-RBD. Beads were then washed four times with RA buffer and resuspended in 50 μl of Laemmlí’s sample buffer. Samples were analyzed by 15% SDS-
PAGE. The separated proteins were transferred to nitrocellulose sheets and probed with monoclonal anti-pan-Ras antibodies (1:500). The immunoreactive bands were visualized by an enhanced chemiluminescence system after incubation with horseradish peroxidase-conjugated anti-mouse IgG.

RESULTS

Stimulation of ERK by ET-1, PDGF, and EGF. ERK activa-
tion was determined by using an anti-active ERK1/2 antibody that recognized the diphotophorylated active form of the enzyme. ET-1, PDGF-BB, and EGF, at supramaximal concen-
trations, stimulated ERK activation in rat myometrial cells, but their kinetics were different (Fig. 1A). For the three agonists, mainly ERK2 phosphorylation was detected. In all cases, the total amount of ERK1/2 was not affected by the different treatments (data not shown). ET-1 stimulated ERK2 phosphorylation in a transient manner with a maximal effect at ~5 min that declined rapidly to almost the basal level within 30 min (Fig. 1A). The time course of PDGF-induced activation of ERK2 also exhibited a peak around 5 min, followed by a sustained phase that persisted for at least 60 min (Fig. 1A). The level of this sustained phase corresponded to ~50% of the peak value (Fig. 1B). In the presence of EGF, the maximal level of ERK2 activation observed after 5 min was equivalent to that obtained with ET-1 and PDGF (Fig. 1, A and B). In contrast to ET-1 and PDGF, EGF-induced ERK2 activation did not decline but remained at the maximal level for at least 60 min (Fig. 1, A and B).

Stimulation of Ras by ET-1, PDGF, and EGF. ET-1 stimulates Ras activation in rat myometrial cells (32). Figure 2A shows that PDGF and EGF also stimulated Ras. For the three agonists, Ras activation was transient with a maximal response at ~3 min. The activation of Ras was more prolonged with EGF than with PDGF. Compared with the two growth factors, ET-1 had a more transient effect. In contrast to the maximal level of ERK activation, which was almost similar among the three agonists, the level of Ras activation differed greatly from one agonist to another: EGF and PDGF were ~10- and 3-fold more efficient than ET-1, respectively (Fig. 2B).

Involvement of PKC in ERK activation. We have previously shown that at least two different PKC isoforms are involved in the ET-1-induced ERK2 activation (32). At least four PKC isoforms (α, δ, ε, and ζ) are expressed in rat myometrial cells (Fig. 3). The β- and γ-isoforms, which were easily detectable in rat brain extracts, were undetectable in myometrial cell extracts (Fig. 3). We investigated the involvement of PKC isoforms in the activation of ERK2 after 5 min of stimulation by PDGF and EGF compared with ET-1. Treatment of the cells by Ro-31-8220, which inhibits all PKC isoforms, almost completely blocked the activation of ERK2 triggered by ET-1, whereas the EGF response was unaffected (Fig. 4A). Interestingly, we observed that the PDGF response, like the ET-1 response, was fully inhibited by Ro-31-8220. Comparable results were obtained with 5 μM bisindolylmaleimide I as well as with 1 μM calphostin C (data not shown).
Characterization of the PKC isoforms involved in ERK activation. Treatment of cells with PMA for 6 h to induce the downregulation of c/nPKC resulted in a complete inhibition of the PDGF response. The ET-1 response was, under these conditions, only partially inhibited (50%), as previously observed (32). As expected, PMA treatment was without effect on the EGF response. Figure 4B shows that treatment of myometrial cells for 6 h with PMA induced the downregulation of PKCα and -δ but not -ε. The ζ-isofrom, being insensitive to phorbol esters, was not affected by PMA treatment. Incubation of the cells for 24 h with PMA, which downregulated PKCε in addition to PKCα and -δ (Fig. 4B), did not increase the inhibition of ET-1-mediated ERK2 activation (Fig. 4A), indicating that PKCε is not involved in this process. Altogether, these results indicated that the EGF-induced ERK activation involves a PKC-independent pathway, whereas activation of ERK by ET-1 and PDGF is fully dependent on PKC. The PDGF response involved mainly c/nPKC, which were downregulated by a 6-h PMA treatment, but not PKCε, which was resistant to this treatment. By contrast, an αPKC, i.e., PKCζ, is partially involved in the ET-1 response. To further characterize the PKC isoforms involved in PDGF- and ET-1-induced ERK activation, we tested the effect of Gö-6976, a PKCα- and PKCβ-selective inhibitor, and rottlerin, a specific PKCδ inhibitor. Gö-6976 and rottlerin, when used at 1 and 10 μM, respectively, have been described to be specific to their respective targets and to be devoid of nonspecific effect on other PKC isoforms (15, 23). Figure 4A shows that in the presence of Gö-6976, the PDGF response was inhibited by ~50% but that the ET-1 response remained unchanged. In contrast, rottlerin reduced by 50% the effect of ET-1 but had no effect on the PDGF response. These data strongly suggest that PKCδ and -ζ participate in the stimulation of ERK by ET-1, whereas the PDGF response is mediated by PKCα and another PMA-sensitive PKC that remains to be defined. This latter PKC was sensitive to a 6-h PMA treatment but resistant to Gö-6976 and rottlerin, indicating that it was different from the PKCα, -δ, and -ε isoforms.

Involvement of PLC in ERK activation. The activation of c/nPKC such as PKCα and -δ requires DAG, a product of PLC activity. The stimulation of PLC by ET-1 and PDGF has already been reported in rat myometrial cells (2, 32). Figure 5A shows that the production of InsPs induced by ET-1 was about
twofold higher than that mediated by PDGF. In contrast, EGF failed to stimulate InsP production (Fig. 5A). We have previously demonstrated in myometrial cells that inhibition of PLC activity by U-73122 abolishes InsP production and partially reduces ERK activation due to ET-1 (32). Similar treatment also blocks PDGF-mediated InsP generation (2). Figure 5B shows that U-73122 strongly inhibited ERK activation triggered by PDGF. In contrast, U-73343, an inactive analog of U-73122, had no effect (Fig. 5B). These results were consistent with the involvement of a PLC- and c/nPKC-dependent pathway in the early (5 min) activation of ERK by PDGF. In contrast, the EGF-induced ERK activation was regulated by a PLC- and PKC-independent process.

**Role of PKC in the sustained phase of ERK activation.** We investigated the involvement of PKC in the sustained phase of ERK2 activation. Figure 6A shows that after 45 min of stimulation, the EGF response remained insensitive to Ro-31-8220. The weak effect of ET-1 after 45 min did not seem to be significantly altered in the presence of Ro-31-8220. Interestingly, Fig. 6A shows that the PDGF-induced ERK2 activation was also insensitive to Ro-31-8220 at 45 min. The stimulation of ERK2 by PDBu, a direct activator of PKC, was inhibited by Ro-31-8220 after 45 min, indicating that the inhibitor remained functional at this time. To obtain more information on PKC dependency of the PDGF response, we tested the effect of Ro-31-8220 on the time course of ERK activation. Figure 6B shows a typical ERK2 activation kinetic plot induced by PDGF with a peak at 5 min and a sustained phase that began after 30 min of incubation. Incubation of cells in the presence of Ro-31-8220 caused a dramatic inhibition of the peak activation (Fig. 6B). With increasing time, the activation of ERK by PDGF became gradually insensitive to Ro-31-8220. This finding indicates that ERK activation triggered by PDGF involves a PKC-independent process that is progressively set up in the later time. Interestingly, the kinetics of the PKC-independent component of the PDGF response were similar to those observed in the presence of EGF (3 ng/ml), which stimulated.
ERK only through a PKC-independent process. Altogether, these results suggest that the activation of ERK by PDGF comprises an acute PKC-dependent phase followed by a PKC-independent sustained phase.

Cooperative effect of ET-1 and EGF on ERK activation and DNA synthesis. ET-1 and PDGF stimulate DNA synthesis in rat myometrial cells in an ERK-dependent manner, as previously demonstrated in the laboratory (2, 32). Figure 7 shows that incubation of the cells for 48 h in the presence of 3 ng/ml EGF led to a 2.5-fold increase in thymidine incorporation that was similar to the maximal ET-1 effect. Increasing the concentration of EGF from 3 to 25 ng/ml did not enhance thymidine incorporation (data not shown). ET-1 and EGF were less efficient (3-fold) than PDGF. This could be correlated to the fact that EGF only induced a sustained ERK activation and ET-1 mainly produced a peak activation, whereas PDGF induced both a peak and a sustained phase. We thus speculated that the coincubation of the cells with ET-1 and EGF would reproduce the effect of PDGF on DNA synthesis. EGF was used at a 3 ng/ml concentration to obtain a sustained phase of ERK activation comparable to that obtained with PDGF (Fig. 6B). At 5 min, EGF (3 ng/ml) had almost no effect by itself but potentiated (2-fold) the ET-1-mediated ERK activation (Fig. 8). By contrast, at 45 min of stimulation, the coincubation of ET-1 with EGF produced the same response as EGF alone. The synergistic effect of ET-1 and EGF was also observed on proliferation. As shown in Fig. 7, treatment of the cells in the presence of EGF and ET-1 produced a 10-fold increase in thymidine incorporation that was similar to that obtained with PDGF (Fig. 7).

DISCUSSION

We previously demonstrated that in rat myometrial cells, the activation of ERK by ET-1 was mediated by at least two PKC isoforms (32). The first one was thought to be PKCζ because it was insensitive to downregulation by PMA treatment and was found to act downstream of PI 3-kinase. Indeed, PKCζ has been described as a PDK1 substrate, a protein kinase activated by PIP3, a product of PI 3-kinase activity (4, 19). The second PKC isoform involved was characterized as PMA sensitive and acted downstream of PLC. The results of the present study indicate that this PKC isoform is PKCβ. Indeed, the inhibition of ERK activation by rottlerin, a specific inhibitor of PKCβ, was identical to that produced after a 6- or 24-h PMA treatment that degraded PKCβ. We previously showed that Src is involved in the stimulation of ERK and that Src activation is PKC dependent (32). The finding that PKCβ and -ζ mediate the effect of ET-1 is consistent with our previous observations because these two PKC isoforms have been shown to interact with Src and to activate it (37, 39). Moreover, it has recently been shown that PKCβ can activate Src through protein tyrosine phosphatase-α (PTPα), which is able to dephosphorylate the inhibitory phosphotyrosine residue located in the COOH-terminal domain of Src (3).

In comparison with ET-1, we investigated the mechanisms by which two growth factors, EGF and PDGF, stimulate the ERK pathway. Both EGF and PDGFβ receptors are receptor tyrosine kinases that stimulate Ras by recruiting the Grb2-Sos complex either directly or through Shc adaptor. The present data demonstrate that EGF is a potent Ras activator that stimulates ERK2 phosphorylation in a PKC-independent manner. This PKC-independent ERK activation induced by EGF has been described in different cell types, including smooth muscle cells (7, 31, 45, 47). In the same context, we found that EGF was unable to stimulate PLC activity. This result was unexpected because it is well established that EGF receptor is able to directly interact with and activate PLCζ. We have already shown (2) that PLCζ is expressed in rat myometrial cells and can be activated in response to PDGF. Nevertheless, this lack of PLC activation by EGF has already been reported by Nojiri et al. (28) in rat hepatocytes in primary cultures.

Fig. 7. Effect of ET-1, PDGF, and EGF on DNA synthesis. Cells were treated in the absence or presence of 50 nM ET-1, 3 ng/ml EGF, or 50 nM ET-1 plus 3 ng/ml EGF for 48 h. [3H]thymidine (2 μCi/ml) was present for the last 24 h. [3H]thymidine incorporation was measured as described in MATERIALS AND METHODS. Results are expressed as cpm/well and are means ± SE of 3 experiments, each carried in duplicate.

Fig. 8. Effect of simultaneous presence of ET-1 and EGF on ERK activation. Cells were treated in the absence or presence of 50 nM ET-1, 3 ng/ml EGF, or 50 nM ET-1 plus 3 ng/ml EGF for 5 or 45 min. The level of ERK2 phosphorylation was analyzed, quantified, and expressed as fold stimulation over basal value. Values are means ± SE of 3 separate experiments.
where EGF receptor and PLCγ1 are expressed. In contrast to the EGF response, we found that PDGF stimulated ERK activation through a PKC-dependent pathway, as already described in other smooth muscle cell systems (22, 29). The present study indicates that in rat myometrial cells, at least two PKC isoforms are involved. The first one was identified as PKCo, an isoform that has been previously shown to be activated by PDGF in rat vascular smooth muscle cells and mesangial cells (5, 13, 36). PKCo-dependent activation of ERK induced by PDGF has been shown to contribute to the mitogenic effect of this growth factor (29). The second PKC isoform can be downregulated by prolonged PMA treatment, but its identity remains to be determined. Although it has been reported that PDGF can activate PKCe in HepG2 cells (27), this isoform does not seem to be involved in the PDGF-induced ERK activation in rat myometrial cells. Indeed, we found that a 6-h PMA treatment was sufficient to produce a full inhibition of ERK activation, whereas it had no effect on PKCe expression level. The high dependency on PMA-sensitive PKC of the PDGF effect is consistent with the result that the PLC inhibitor U-73122 also blocked PDGF-induced ERK activation. It is intriguing to find that ET-1, PDGF, and EGF receptors, which are generally considered potential activators of the PLC/PKC pathway, use divergent mechanisms to stimulate ERK phosphorylation at early times. One possible explanation resides in the localization of receptor and associated signaling cascades in specialized membrane microdomains such as caveolae or rafts. Several receptors, including the ET-1, PDGF, and EGF receptors, have been shown to be localized in caveolae and rafts (20, 24, 34, 46). This localization seems to depend on the cell type and the activation status of the receptors. Rybin et al. (34) have shown that, in cardiomyocytes, ET-1 can recruit specific PKC isoforms to caveolae where Raf-1, MEK, and ERK1/2 are present. We speculate that, in rat myometrial cells, the receptors for ET-1, PDGF, and EGF are differentially localized in such microdomains. On the other hand, the absence of EGF-mediated InsP production may also reflect that EGF receptors and PIP2 are associated with different membrane microdomains. This hypothesis is supported by the observation that, in A431 epidermoid carcinoma cells, PIP2 is localized in caveolae (44), whereas the EGF receptor-rich plasma membrane fraction is clearly distinct from caveolae (43).

Altogether, our results support the notion of complementary functions between Ras and PKC pathways. It seems that, depending on its level of activation, Ras is not always sufficient to maximally induce the early phase of ERK activation. ET-1 and PDGF, which moderately stimulate Ras, utilize a PKC-dependent pathway in addition to the Ras pathway to fully stimulate ERK activation at 5 min. Conversely, EGF, which highly stimulates Ras, does not need a PKC-dependent pathway to maximally activate ERK. In contrast to the early phase of ERK activation, the sustained phase is a PKC-independent process for the three agonists. The amplitude of the sustained phase of ERK activation rather correlates with the level and persistence of Ras activation. The existence of a PKC-dependent transient phase followed by a PKC-independent sustained phase has also been described in Rat-1 fibroblasts stimulated by lysophosphatidic acid (6). By contrast, in Chinese hamster ovary cells expressing sst receptors, somatostatin stimulates ERK through a rapid Ras-dependent/PKC-independent pathway followed by a sustained phase mediated by a PKC-dependent pathway (38).

The decrease of ERK activity following the initial peak has been attributed to phosphatases that dephosphorylate ERK. MAP kinase phosphatases (MKP) are dual-specificity phosphatases that closely control the level of ERK activation and are involved in the inactivation of ERK. Although MKP-1 gene transcription has been described as being activated by the ERK pathway (30), some reports indicate that it also can be activated by a PKC-dependent but ERK-independent mechanism (25, 40, 42). This latter mechanism does not seem to be involved in ERK activation in myometrial cells with the three agonists used during the present study, because we observed that the level of the sustained phase was insensitive to PKC inhibitors.

The results of DNA synthesis experiments indicate that PDGF is the most potent agonist for proliferation; ET-1 and EGF have similar efficiency corresponding to about one-third the PDGF effect. The biphasic kinetics, observed with PDGF, composed of a transient activation followed by a sustained but lower level of ERK activity, are a common feature of cell proliferation in many systems (30). This may explain the lower effect of EGF and ET-1, because they induce only a monophasic ERK activation. Interestingly, when myometrial cells were coincubated with ET-1 and EGF, the mitogenic effect was potentiated and became as high as that of PDGF. Under these conditions, the two monophasic responses of ET-1 and EGF
are combined to obtain a biphasic profile of ERK activation comparable to that observed with PDGF. These observations are corroborated by those obtained in different cell systems, where G protein–coupled receptor agonists potentiated the mitogenic effect of growth factors in smooth muscle cells (9, 11, 12). In human uterine leiomyoma cells, this potentiation was shown to be mediated by c/nPKC (11). The synergistic effect between ET-1 and EGF could be associated with a transactivation of the EGF receptor by ET-1 (8). However, this mechanism is unlikely in rat myometrial cells because the activation of ERK by ET-1 does not involve the EGF receptor tyrosine kinase activity (32).

In conclusion, our results demonstrate that proliferation of rat myometrial cells is controlled by a complex network of signaling pathways (Fig. 9) involving PKC-dependent and -independent processes coupled to G protein–coupled receptors as well as to receptor tyrosine kinases. This network regulates the temporal evolution of ERK activity, resulting in a subtle tuning of the proliferative status of myometrial cells that play a critical role in the uterine physiological function.

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