ET-1 stimulates ERK signaling pathway through sequential activation of PKC and Src in rat myometrial cells

PHILIPPE ROBIN,* ISALINE BOULVEN,* CHRISTINE DESMYTER, SIMONE HARBON, AND DENIS LEIBER
Laboratoire de Signalisation et Régulations Cellulaires, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8619, Université de Paris-Sud, 91405 Orsay cedex, France

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phosphoinositide 3-kinase; deoxyribonucleic acid synthesis; phospholipase C; Ras

Endothelin (ET)-1 is a peptidic hormone described first as a potent vasoconstrictor and later as an important physiological modulator of uterine activities. Indeed, ET-1 is synthesized in intrauterine tissues (40) and can affect myometrial cells in a paracrine fashion to induce contractility. In rat myometrium, our laboratory previously showed (23) that ET-1 activates the ET<sub>A</sub> receptors that are functionally coupled to G<sub>q/11</sub> protein to stimulate phospholipase C (PLC) activity and to G<sub>i</sub> protein to inhibit adenylyl cyclase. The increase in Ca<sup>2+</sup> concentration induced by inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) and a decrease of the cAMP level are the major determinants of ET-1-induced uterine contraction. Additional data from our laboratory indicate that ET-1 also activates protein tyrosine kinases that contribute to stimulation of rat uterine contractility (38) and protein kinase C (PKC) (29), which has been involved in human uterine contractility at term (13).

ET-1 also has mitogenic properties in various cell types, including human myometrial cells (2, 8). It is well established that the initiation of proliferation relies on the activation of the mitogen-activated protein (MAP) kinase cascade (18). The activation of MAP kinases has been observed in human myometrial cells stimulated by interleukin-1 and oxytocin (1, 37), in nonpregnant rat myometrial cells on platelet-derived growth factor (PDGF) stimulation (6), and in puerperal rat myometrial cells in response to G protein-coupled receptors activation by ET-1, prostaglandin F<sub>2α</sub>, and oxytocin (25, 35, 36). The mechanisms involved in the stimulation of this signaling pathway by G protein-coupled receptors are complex and seem to vary with the cell type and agonist used. These mechanisms may require the transactivation of a receptor tyrosine kinase such as epidermal growth factor (EGF) receptor or stimulation of nonreceptor tyrosine kinases including Src family kinases (18, 39). The activation of these tyrosine kinases results in the recruitment of the adaptor protein Grb2 on tyrosine phosphorylated proteins. Grb2 is associated with Sos, the specific exchange factor of Ras, which catalyzes the conversion of inactive Ras-GDP into active Ras-GTP. Activated Ras initiates...
the MAP kinase cascade constituted by the kinases Raf-1, MAP kinase kinase (MEK), and extracellular signal-regulated kinases (ERKs). Such a mechanism, mediated by transactivation of the EGF receptor tyrosine kinase, has been observed in vascular smooth muscles cells (21) and in human ovarian carcinoma cells (52) stimulated by ET-1.

A role for PKC has also been described in the ERK activation process induced by ET-1 (5, 20, 22, 54). PKC is a family of serine/threonine kinases that is subdivided into three groups on the basis of structural and biochemical properties, conventional (c; α, β1, β2, γ), novel (n; δ, ε, η, θ, ι, ν) and atypical (α; ζ, ξ, η/λ) isoforms. c/nPKC isoforms are activated by diacylglycerol (DAG), a product of phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis, and by phorbol esters. In addition to DAG, cPKC requires Ca2+ for full activation (41). In contrast to c/nPKC, aPKCs are insensitive to DAG and are activated by other pathways. Indeed, PKC-ζ is stimulated through phosphorylation by phosphoinositide (PI)-dependent protein kinase (PDK) 1 in a phosphatidylinositol 3,4,5-trisphosphate (PIP3)-dependent manner (10, 28). This signaling pathway is activated by PI 3-kinase, which catalyzes the phosphorylation of PIP2 into PIP3. The mechanisms by which PKC could activate the MAP kinase cascade are not fully elucidated and seem to be, in some extent, cell type specific. It has been reported that PKC activates Raf-1 (26) or MEK (34) through a direct phosphorylation. However, PKC may also stimulate Ras through the activation of protein tyrosine kinases (3, 14).

Given that proliferation is one of the main functions of myometrial cells in uterine physiology, we aimed to investigate whether ET-1 is able to induce proliferation of rat myometrial cells and to determine the mechanisms involved. Because ERKs are essential for mitogen-induced DNA synthesis, we focused our study on the characterization of the pathways controlling ERK activation in response to ET-1. The results presented here highlight the crucial role of the sequential activation of PKC and Src family tyrosine kinases in this process.

MATERIALS AND METHODS

Materials. ET-1 and FR-139317 were from NeoSystem (Strasbourg, France), and PDGF-BB was from Peprotech (Tebu, Le Perray-en-Yvelynes, France). EGF, wortmannin, pertussis toxin (PTX), β-estradiol-3-benzoate, leupeptin, aprotinin, LiCl, phorbol 12-myristate 13-acetate (PMA), and phenol 12,13-dibutyrate (PDBu) were from Sigma (St. Louis, MO). Western blotting detection reagents and [γ-32P]ATP were obtained from Du Pont New England Nuclear Products Division (Paris, France). Myo-[2-3H]inositol (10–20 Ci/mmol), pGEX-2T vector, and glutathione-Sepharose CL-4B were obtained from Amersham Pharmacia Biotechnology (Les Ulis, France). PP1, PP3, PD-98059, U-73122, and U-73342 were from Bioworld (Plymouth Meeting, PA). Tyrphostins AG-1296 and AG-1478, Ro-31–8220, sarafotoxin S6c, BQ-788, LY-294002, and U-0126 were from Calbiochem (Los Angeles, CA). Collagenase was from Boehringer Mannheim (Meylan, France). Polyclonal antibody to ERK1/2 was from Zymed Laboratories (San Francisco, CA), and polyclonal anti-ERK1/2 antibody was from Promega (Madison, WI). Monoclonal anti-Src antibody (clone GD11), monoclonal anti-Ras antibody, and Src substrate peptide were from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated anti-rabbit antibodies were from Dako (Trappes, France). Protein G Plusagarose was from Santa Cruz (Tebu, Le Perray-en-Yvelynes, France). All other reagents were of the highest grade commercially available.

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

Myometrial cell preparation and culture. Primary cultures of myometrial cells were prepared by collagenase digestion as previously described (6). 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 at a plating density of 3.5×104 cells/ml. The medium was changed every 2 days, and the cells were kept in serum-free medium for 48 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). The cells were washed twice with Hanks’ balanced salt solution containing 20 mM HEPEs (pH 7.5) and then incubated at 37°C in fresh buffer with 10 mM LiCl. After 10 min, the agents to be tested were added at the indicated concentrations and incubation was 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). The 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 (6). Results were expressed as counts per minute per well.

Western blot analysis of phosphorylated ERK1/2. Serum-starved confluent myometrial 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 the addition of 100 µl of cold solubilization buffer (50 mM HEPEs, pH 7.4, 150 mM NaCl, 100 mM NaF, 10% glycerol, 10 mM Na3P2O7, 200 µM Na3VO4, 10 mM EDTA, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride (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 sample buffer and analyzed by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose sheets and probed with polyclonal anti-ERK1/2 antibodies (1:5,000). The blots were 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).

**[^3H]thymidine incorporation.** Serum-starved myometrial cells (50% confluent) in 24-well dishes were incubated with the various agents to be tested for 24 h, and then[^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% w/vol). Radioactivity incorporated into TCA-precipitable material was recovered with 0.5 ml of 1 N NaOH and quantified by liquid scintillation counting.

**Measurement of Src kinase activation.** Src activity was determined as previously described (7). Serum-starved confluent myometrial cells seeded in 55-cm² petri dishes 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 stimulated by incubation for 3 min with 50 nM ET-1. Reactions were stopped by aspiration of the incubation medium followed by addition of 500 μl of cold solubilization buffer. Cells were detached by scraping on ice and were centrifuged at 10,000 g for 10 min at 4°C. For immunoprecipitation, the supernatants were each incubated with 2 μg of monoclonal anti-Src antibodies for 1 h at 4°C. We then added 20 μl of protein G Plus-Agarose, and the incubation was continued for another 1 h. The immunoprecipitates were washed four times in solubilization buffer and once in kinase buffer [50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol (DTT), and 100 μM Na₃VO₄] Src activity was assayed at 30°C for 20 min in 50 μl of kinase buffer supplemented with 100 μM Src substrate peptide (KVEKIEGTGYVYVK) (9) and 50 μM [γ-[^32]P]ATP (5 μCi). The reaction was stopped by adding 25 μl of 50% acetic acid. The samples were centrifuged, and the supernatants were spotted onto phosphocellulose paper strips. The strips were washed in 0.4% H₃PO₄ three times for 15 min each and once in acetone and then dried. The dried strips were counted for radioactivity in the presence of scintillation fluid.

**Ras activation assay.** The DNA fragment coding for amino acids 51–131 of the Ras binding domain (RBD) of human c-Raf-1 was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from human cDNA with the following set of primers: forward 5’-CACAGATGATTCTCAAGACACAAGCACACAC-3’; reverse 5’-GGGAAAGATTCAGGAAATCTAC-3’. The forward primer contains a BamHI restriction site that allows the in-frame insertion of the RBD coding sequence with the glutathione-S-transferase (GST) gene located in the pGEX-2T vector. The reverse primer was designed to introduce a stop codon followed by an EcoRI restriction site. The RT-PCR product was digested with BamHI and EcoRI restriction enzymes and inserted in the BamHI-EcoRI sites of pGEX-2T plasmid. The plasmid obtained (pGEX-RBD) was sequenced to verify good insertion of the RBD coding sequence and then introduced in *Escherichia coli* BL21 cells for production of the fusion protein GST-RBD. 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. The beads were then washed five times in the same buffer.

Ras activation was determined by Ras-GTP pull-down assay with GST-RBD. Serum-starved confluent myometrial cells seeded in 55-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 Na₂PO₄, 200 μM Na₃VO₄, 10 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5 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 final volume of 2 ml. Beads were then washed four times with RA buffer and resuspended in 50 μl of Laemmli sample buffer. Samples were analyzed by 15% SDS-PAGE, and 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.

**Data analysis.** Results are expressed as means ± SE and were analyzed statistically with Student’s t-test. A P value of ≤0.05 was considered significant.

**RESULTS**

**Activation of ERK is involved in mitogenic effect of ET-1.** Treatment of rat myometrial cells in primary culture with ET-1 enhanced incorporation of[^3H]thymidine (Fig. 1). DNA synthesis triggered by ET-1 was strongly reduced when the cells were pretreated with PD-98059, a selective inhibitor of MEK. This result pointed out the critical role played by the ERK cascade in the mitogenic effect of ET-1. Data in Fig. 2A show that ET-1 induced ERK2 activation. ET-1-mediated activation of ERK2 was time dependent (Fig. 2B), with a maximal response at 5 min that slowly declined to a low but observable level that persisted for at least 60 min.
The stimulatory effect of ET-1 was dose dependent (EC_{50} = 1–2 nM), with a maximal effect at 10 nM (Fig. 2C). Stimulation of ERK2 triggered by ET-1 was potently reduced after incubation of the cells with PD-98059 (Fig. 2A). The present observations demonstrate that ET-1 exerts a mitogenic effect on rat myometrial cells that is consecutive to the activation of ERK.

Treatment of cells with FR-139317, a specific antagonist of ET_{A} receptors, strongly reduced ERK2 activation induced by ET-1, whereas BQ-788, a specific antagonist of ET_{B} receptors, had only a small effect (~15% inhibition), indicating that ET-1-induced ERK2 activation is mainly due to ET_{A} receptor activation (Fig. 2D). Consistent with these observations, stimulation of cells with S6c, a specific agonist of ET_{B} receptors, only induced a small activation of ERK2 (15–20% of ET-1 response; Fig. 2D). This minor S6c effect was completely blocked by BQ-788 but resistant to FR-139317 (Fig. 2D).

**ET-1-induced ERK2 activation is not due to transactivation of EGF and PDGF receptors.** Some reports have demonstrated that activation of ERK by ET-1 could occur through transactivation of a growth factor receptor such as EGF receptor (21, 52). We recently demonstrated (6) that PDGF-BB also stimulated ERK2 activity in our cell system, and this response was completely blocked by AG-1296, a selective inhibitor of

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**Fig. 2.** Stimulatory effect of ET-1 on extracellular signal-regulated kinase (ERK) activation. A: myometrial cells were incubated in the absence (control) or presence of 50 nM ET-1 for 5 min without or with 20 µM PD-98059 (added 20 min before ET-1). Detergent-extracted proteins were analyzed by 10% SDS-PAGE. Immunoblotting (IB) was performed with anti-active ERK1/2 antibodies (top) or with anti-ERK1/2 antibodies (bottom). Data are from 1 of 3 experiments. B: cells were treated with 50 nM ET-1 for the times indicated. Detergent-extracted proteins were analyzed by 10% SDS-PAGE and immunoblotting with anti-active ERK1/2 antibodies. Blots were stripped and reprobed with anti-ERK antibody. Phosphorylated ERK2 (pERK2) and total ERK2 were quantified with a densitometer (Molecular Dynamics). The level of ERK2 phosphorylation was normalized with respect to total ERK2 amount in each sample and expressed as %maximal response. Values are means ± SE of 3 separate experiments. C: cells were treated for 5 min with the indicated ET-1 concentrations, and the detergent-extracted proteins were analyzed as in B. Results are expressed as %maximal response. Values are means ± SE of 3 separate experiments. D: cells were pretreated without or (for 20 min) with 100 nM BQ-788 or 1 µM FR-139317 and then stimulated for 5 min with either 50 nM ET-1 or 50 nM sarafotoxin S6c. The level of ERK2 phosphorylation was determined by immunoblotting as described above and expressed as %maximal ET-1 response. Values are means ± SE of 3 separate experiments. *P < 0.05 vs. ET-1 alone; #P < 0.05 vs. S6c alone.
PDGF receptors (Fig. 3). EGF also stimulated ERK2 activation in myometrial cells, and this response was abolished in the presence of AG-1478, a specific inhibitor of the tyrosine kinase of EGF receptors (Fig. 3). In contrast, ERK2 activation triggered by ET-1 was completely insensitive to these two inhibitors (Fig. 3). Thus the activation of ERK2 induced by ET-1 was mediated by biochemical mechanisms that do not involve either PDGF or EGF receptor tyrosine kinase.

ERK2 activation by ET-1 is mediated by PKC and PLC activation. Phosphorylation of ERK2 in response to ET-1 was abolished by pretreatment of cells with Ro-31–8220, a specific inhibitor of all classes of PKC (Fig. 4A). Similar inhibition was obtained with 5 μM calphostin C and 5 μM bisindolylmaleimide I, two other PKC inhibitors (data not shown). Downregulation of c/nPKC by incubation of cells with 1 μM PMA for 6 h also attenuated the activation of ERK2 induced by ET-1, but to a lesser extent (48%; Fig. 4A). When the incubation in the presence of PMA was lengthened to 20 h, the level of inhibition was not increased (data not shown). PDBu also stimulated the phosphorylation of ERK2, which, as expected, was completely inhibited by Ro-31–8220 and by c/nPKC downregulation (Fig. 4A).

Stimulation of c/nPKC is generally consecutive to PLC activation that produces DAG and InsP3. Data shown in Fig. 4B demonstrate that incubation of rat myometrial cells with ET-1 stimulated the production of [3H]inositol phosphate (InsP). In some experiments, cells were incubated or not for 20 min with 1 μM FR-139317 (FR) or 100 nM BQ-788 before stimulation with 50 nM ET-1 or S6c. Results are expressed as cpm/well (×10−3) and are means ± SE of 3 separate experiments, each performed in duplicate. C: cells were pretreated for 20 min without or with 2 μM U-73122 or 2 μM U-73343 and then stimulated with 50 nM ET-1 for 5 min. The level of ERK2 phosphorylation was quantified as in Fig. 2 and expressed as %maximal ET-1 response. Values are means ± SE of 5 separate experiments. *P < 0.05 vs. both control and ET-1 alone.

Fig. 3. ET-1-mediated ERK2 activation does not involve the transactivation of either the platelet-derived growth factor (PDGF) or the epidermal growth factor (EGF) receptor. Cells were incubated for 20 min without or with 5 μM AG-1296 or 2 μM AG-1478 and then stimulated for 5 min with 50 nM ET-1, 25 ng/ml PDGF-BB, or 50 ng/ml EGF. Detergent-extracted proteins were analyzed by 10% SDS-PAGE and immunoblotting with anti-active ERK1/2 antibodies. Data are from 1 of 3 separate experiments.

Fig. 4. ET-1 stimulates ERK2 via inositol phosphate (InsP) production and protein kinase (PKC). A: cells were pretreated without or with 5 μM Ro-31–8220 for 20 min or 1 μM phorbol 12-myristate 13-acetate (PMA) for 6 h and then stimulated for 5 min with either 50 nM ET-1 or 1 μM phorbol 12,13-dibutyrate (PDBu). The level of ERK2 phosphorylation was determined by immunoblotting as described in Fig. 2 and expressed as %maximal ET-1 response. Values are means ± SE of 5 separate experiments. *P < 0.05 vs. ET-1 alone; #P < 0.05 vs. PDBu alone; †P < 0.05 vs. both ET-1 alone and ET-1 + Ro-31–8220. B: [3H]inositol-labeled myometrial cells were incubated for 10 min in the presence of the indicated concentrations of ET-1. Total [3H]InsPs was determined as described in MATERIALS AND METHODS. In some experiments, cells were incubated or not for 20 min with 1 μM FR-139317 (FR) or 100 nM BQ-788 before stimulation with 50 nM ET-1 or S6c. Results are expressed as cpm/well (×10−3) and are means ± SE of 3 separate experiments, each performed in duplicate. C: cells were pretreated for 20 min without or with 2 μM U-73122 or 2 μM U-73343 and then stimulated with 50 nM ET-1 for 5 min. The level of ERK2 phosphorylation was quantified as in Fig. 2 and expressed as %maximal ET-1 response. Values are means ± SE of 5 separate experiments. *P < 0.05 vs. both control and ET-1 alone.
of InsPs. The response was dose dependent (EC50 = 1–2 nM), with a maximal effect obtained at 10 nM. This dose-dependent stimulation of PLC activity is very similar to that of ERK induced by ET-1, suggesting a close relationship between these two phenomena. Incubation of cells with FR-139317 produced almost total inhibition of ET-1 response, whereas incubation with BQ-788 had no effect (Fig. 4B). Moreover, stimulation of cells with S6c very weakly increased InsP production (Fig. 4B). These results demonstrate that ET-1 stimulates InsP production through ET A receptors. Incubation of the cells in the presence of U-73122, a selective inhibitor of PLC activities, partially reduced ERK2 activation due to ET-1 (Fig. 4C). In contrast, treatment of the cells with U-73343, the inactive analog of U-73122, failed to modify the activation of ERK2 (Fig. 4C) triggered by ET-1. We verified that U-73122 abolished the production of InsPs induced by ET-1, whereas U-73343 had no effect (data not shown). The data indicate that the ET-1-induced ERK2 activation in rat myometrial cells is consecutive to the activation of PLC and PKC. The low inhibitory effect due to c/nPKC downregulation and PLC inhibition, compared with the total inhibition obtained with Ro-31–8220, may reflect the involvement of an aPKC, which is insensitive to DAG and phorbol esters, in ERK activation induced by ET-1.

**ET-1-stimulated ERK2 involved a PI 3-kinase- and PTX-sensitive G protein-dependent pathway.** It has been reported that PKC-ζ, an aPKC, is activated by a PI 3-kinase-dependent pathway (10, 34, 44, 50). We therefore analyzed the effect of wortmannin, an inhibitor of PI 3-kinase, on ET-1-induced ERK2 activation. Treatment with wortmannin inhibited ET-1-dependent ERK2 activation by 54 ± 3% (Fig. 5A). A similar partial inhibition (53 ± 11%) was obtained when the cells were incubated in the presence of 20 μM LY-294002, another PI 3-kinase inhibitor. Treatment of cells with PTX also partially reduced ERK activation (52 ± 5% inhibition). This effect of PTX was not additive with that produced by wortmannin (Fig. 5A), indicating that PI 3-kinase and G1 protein act in a common pathway to trigger ERK2 activation in response to ET-1.

We also tested the combined effect of wortmannin and PKC downregulation on ET-1-induced ERK2 activation. As shown in Fig. 5B, the effects of both wortmannin and PMA treatment, which were completely additive, resulted in total inhibition. These data indicated that PLC-c/nPKC and G1-PI 3-kinase pathways act in parallel to activate ERK2. These results reinforce the hypothesis of the involvement of an aPKC in the latter pathway.

**ET-1 stimulation of ERK2 involved activation of Src.** Treatment of the cells with PP1, a specific inhibitor of Src, abolished ERK2 activation induced by ET-1 (Fig. 6A). PP3, the inactive analog of PP1, failed to reduce the stimulatory effect of ET-1 (Fig. 6A). Interestingly, activation of ERK2 stimulated with PDBu was also potently inhibited by PP1 (Fig. 6A), indicating that Src may act downstream of PKC. To confirm this observation, we tested the ability of ET-1 and PDBu to stimulate Src activity. The results in Fig. 6B show that ET-1 and PDBu are both able to activate Src about twofold. Moreover, Ro-31–8220 inhibited the activa-
tion of Src stimulated by ET-1 and PDBu, confirming that, in both cases, Src activation is consecutive to PKC activation.

**ET-1 stimulated Ras activation.** The potency of ET-1 to activate Ras was investigated with a GST fusion protein including the RBD of Raf. ET-1 caused a rapid but transient activation of Ras that peaked at 3 min (Fig. 7A). Activation of Ras by ET-1 was inhibited when the cells were treated with PP1 and Ro-31–8220, indicating that the PKC-Src pathway was required for this process (Fig. 7B).

**DISCUSSION**

In the present study, we demonstrated that ET-1 is able to stimulate DNA synthesis in rat myometrial cells in primary culture through the activation of ERK pathway. Considering the heterogeneity of the mechanisms involved in ERK activation by G protein-coupled receptors, we characterized, in rat myometrial cells, the different pathways operating in ET-1-dependent ERK activation.

It is well documented that G protein-coupled receptors may activate ERK through the stimulation of tyrosine kinases including growth factor receptors such as EGF or PDGF receptors or nonreceptor tyrosine kinases. In this context, it has been reported in vascular smooth muscle cells and ovarian carcinoma cells that ET-1 transactivates the EGF receptor to stimulate ERK signaling pathway (21, 52). In rat myometrial cells, we found that ERK activation was independent of either the EGF or the PDGF receptor but involved a nonreceptor tyrosine kinase of the Src family. Indeed, we found that ET-1 stimulated Src activity whose inhibition abolished ERK activation. These data are in line with various reports indicating that ET-1 is able to stimulate Src family tyrosine kinases, which may mediate ERK activation (4, 11, 49).

In other respects, we demonstrate here the major role played by PKC in the signaling pathway triggered by ET-1 in rat myometrial cells. Indeed, inhibition of PKC abrogated ERK activation, which is consistent with previously published data (20, 32, 54). Interestingly, PKC inhibition also abolished Src activation induced by ET-1, suggesting that Src is a downstream effector of PKC. This notion was strengthened by the result that direct activation of PKC by a phorbol ester...
similarly stimulated Src activity. Recent reports also indicate that Src acts downstream of PKC in signaling pathways triggered by gonadotropin-releasing hormone (GnRH) (3, 15, 30). However, the mechanisms by which PKC could activate Src are not clearly established. As indicated by recent studies, PKC-δ, ε, and ζ are able to interact directly with Src family kinases, increasing the catalytic activity of the tyrosine kinases (46–48, 53). Activation of Src by PKC may also occur through focal adhesion kinase family kinases such as Pyk2. Indeed, PKC, in conjunction with Ca^{2+}, has been shown to activate Pyk2 (17, 42), which in turn may recruit and activate Src (12). Whatever the mechanism involved in the stimulation of Src by PKC, our data demonstrated that the sequential activation of PKC and Src is required for ET-1-induced ERK activation.

Src-dependent activation of ERK generally involves a Ras-dependent pathway (12, 31, 43, 45), which occurs through the tyrosine phosphorylation of Shc and recruitment of the Grb2-Sos complex. In rat myometrial cells, we found that ET-1 induced a transient activation of Ras. The stimulation of Ras was dependent on Src activity and, as expected, on PKC activity. Together, our data suggest that the sequential activation of PKC and Src by ET-1 leads to ERK stimulation in a Ras-dependent manner. However, previously published data show that PKC-dependent activation of ERK may occur via a direct activation of Ras (26, 33, 54) or MEK (34, 50) by PKC. We therefore cannot exclude that such processes could influence the Ras-dependent ERK activation. In this context, Benard et al. (3) showed that, in αT3–1 cells, GnRH stimulated ERK activation through both PKC-Src-Ras-Raf and PKC-Raf pathways. However, in contrast to what we found in myometrial cells, the direct activation of Raf by PKC appears to be the main pathway in this cell model.

In human myometrial cells, Eude et al. (13) demonstrated that ET-1-induced proliferation was totally dependent on cPKC-α. In rat myometrial cells, we demonstrated that c/nPKCs, activated through a G_{q}-PLC-β pathway, are involved in ERK activation but only for ~50%. The remaining 50% have been attributed to an aPKC, possibly PKC-ζ. This isoform is expressed in rat myometrium (24) and is a substrate for PKD1, a kinase regulated by PIP_3, a product of PI 3-kinase activity (10, 34). A role for PI 3-kinase in ERK activation by G protein-coupled receptors, including lysophosphatidic acid, angiotensin II, and ET receptors, has been described (4, 16, 50). In rat myometrial cells, we showed that ET-1-induced ERK activation is partially (50%) dependent on PI 3-kinase activity, strengthening the hypothesis of the involvement of aPKC. We also demonstrated that the PI 3-kinase-dependent activation of ERK by ET-1 was sensitive to PTX. This is consistent with previous studies that demonstrated the existence of a G_{i}-PI 3-kinase pathway in the activation of ERK by diverse G protein-coupled receptors such as lysophosphatidic acid and e_{2}-adrenergic receptors (19, 50).

Together, our results allow us to propose a model for ET-1-induced ERK activation and DNA synthesis (Fig. 8) in which ET-1, mainly through ETA receptors, activates two parallel pathways that contribute equally to ERK activation. The first pathway involves G_{q}, PLC-β, and c/nPKC and the second pathway G_{i}, PI 3-kinase, and aPKC. Activated PKCs in turn trigger the sequential stimulation of Src and Ras, leading to ERK activation. This model is compatible with previous results from our laboratory (23) that indicated that in intact myometrium, ET-1, through ETA receptor subtype, activates both PTX-sensitive and -insensitive G proteins, the latter being coupled to PLC-β activation.

Kimura et al. (25) described in rat puerperal myometrial cells that PTX blocked ET-1-mediated ERK activation and that PMA-sensitive PKC was not involved in this regulatory pathway. In myometrial cells from prepubertal rats, we also demonstrated that stimulation of ERK by ET-1 was regulated through the activation of a PTX-sensitive, PMA-insensitive process, suggesting the existence of a common pathway in the two cell models. Moreover, Kimura et al. (25) showed the involvement of Sos, the exchange factor of Ras, the latter being activated in our model. However, in myometrial cells from prepubertal rats, the PTX-sensitive pathway contributes only 50% to the stimulation of ERK triggered by ET-1, the remaining 50% being attributed to the G_{q}, PLC, and c/nPKC pathway. At the end of gestation in puerperal myometrial cells, this last pathway no longer contributes to ERK activation but is devoted to the activation of other components of the
contractile machinery (25). This suggests that signaling pathways leading to ERK activation in response to ET-1 are altered during gestation. This is consistent with previous data from our laboratory (27, 51) showing that the expression and/or functions of G proteins and effectors such as PLC are the target for differential regulation, linked to the hormonal environment during gestation. Together, these data illustrate the complexity of the mechanisms of regulation of ERK, which may depend not only on the stimulus and the cell type but also on the physiological status of the tissue.

In summary, we present evidence that in rat myometrial cells, ET-1, which is an important physiological modulator of uterine activities, stimulates DNA synthesis. This occurs through the sequential activation of PKC, Src, and Ras, which leads to ERK activation. Our data strongly suggest that c/nPKC and aPKC, which are activated downstream of PLC and PI 3-kinase, respectively, contribute equally to ET-1-induced ERK activation. The differential regulation of these signaling pathways may be of physiological relevance for the precise coordination, from implantation to parturition, of the two major functions of uterine smooth muscle, namely, contraction and proliferation. The characterization of these diverse regulatory pathways is of particular interest in light of the importance of cell proliferation in the physiology of the myometrium during gestation. Moreover, deregulation of the pathways controlling cell proliferation is undoubtedly determinant for the presence of pathological states of myometrial cells such as leiomyoma.

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