Serotonin-induced MMP-13 Production Is Mediated via Phospholipase C, Protein Kinase C, and ERK1/2 in Rat Uterine Smooth Muscle Cells

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Serotonin (5-hydroxytryptamine; 5-HT), acting via the 5-HT2A receptor, up-regulates the transcription and production of interstitial collagenase (matrix metalloproteinase-13; MMP-13), a critical enzyme responsible for maintaining the integrity of the uterus, after parturition. Serotonin treatment of rat uterine myometrial smooth muscle cells induced inositol phosphate (IP) turnover, which was abolished by the 5-HT2A receptor-specific antagonists ketanserin and spiperone. The phospholipase C (PLC) inhibitors U73122 and D609 attenuated serotonin-mediated-IP turnover with a corresponding inhibition of MMP-13 protein production. Subsequent recovery of both MMP-13 protein expression and IP generation was seen following the removal of D609. Protein kinase C (PKC) activators, the diacylglycerol analogue 1,2-dioctanoyl-sn-glycerol and phorbol myristate acetate (PMA), mimicked the effect of serotonin on MMP-13 protein expression; prolonged PMA treatment (which down-regulates PKC) lowered MMP-13 protein levels. The PKC-specific inhibitors bisindolylmaleimide I, calphostin C, CGP 41251, and the MMP-13 protein levels. The PKC-specific inhibitors bisindolylmaleimide I, calphostin C, CGP 41251, and the PKCβ-selective inhibitor rottlerin were able to suppress serotonin up-regulation of MMP-13. Furthermore, the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 blocked serotonin-dependent activation of p44/42 MAPK (pERK1/2), a downstream effector of PKC and also down-regulated MMP-13 protein expression. Similarly, calphostin C and rottlerin depressed activation of p44/42 MAPK. From these studies, serotonin, binding through the 5-HT2A receptor, initiates a signaling cascade whereby stimulation of PLC leads to the activation of PKC and subsequently the ERK1/2 pathway, which ultimately results in MMP-13 production.

The maintenance of the three-dimensional architecture of the mammalian uterus is carefully regulated during pregnancy and after parturition. Production of fibrillar collagens (Types I and III) is up-regulated 10-fold in the uterus during pregnancy (1, 2). These collagens possess a rigid superhelical structure, which is responsible for providing the tensile strength necessary to accommodate the force exerted by the growing fetus. After parturition, a precisely programmed non-necrotic and non-inflammatory process of connective tissue remodeling occurs, which results in the rapid and dramatic degradation of the collagen amassed and returns the uterus to reproductive competence (3). This transition is initiated by the enzyme interstitial collagenase (matrix metalloproteinase-13; MMP-13), a member of the matrix metalloproteinase family. It acts as not only the rate-limiting step but also the committed step in the catalysis of the degradation of extracellular collagen (4, 5). The expression of MMP-13 occurs during postpartum involution and is not present either during the pregnancy or even within hours prior to parturition. The myometrial smooth muscle cell of the uterus has been identified not only as the source for MMP-13 (6) but also that of its natural substrate, collagen (7). Thus, the myometrial smooth muscle cell provides an interesting paradigm for not only the synthetic needs of the uterus during pregnancy but also the degradative events that occur postpartum.

Our laboratory has focused on MMP-13 and the regulatory mechanisms involved in the restructuring of the postpartum uterus by this critical enzyme. To this end, we have developed a primary cell culture system with rat myometrial smooth muscle cells (SMCs) that produce MMP-13 (8, 9). We have found that the indoleamine serotonin (5-hydroxytryptamine, 5-HT) is an obligate inducer of MMP-13 in both rat and human myometrial SMCs (8, 10). Nuclear run-on analysis demonstrated that serotonin treatment in rat myometrial SMCs increased MMP-13 gene transcription by 6-fold, which corresponded to maximal steady-state mRNA and protein production following exposure to serotonin (11). Additionally, we have determined that interleukin-1 (IL-1) is induced in a serotonin-dependent manner and is itself required for the production of MMP-13 (12). The requisite IL-1 presence acts to not only induce autocrine expression of the genes for IL-1α and β but also that of transin (rat stromelysin) and 92 kDa gelatinase (type IV collagenase), as well as the gene for IL-6.

Interestingly, we have also found that serotonin acts to down-regulate the production of genes responsible for the hypertrophic nature of the uterus during pregnancy. Serotonin exerts negative regulatory effects upon the genes for types I and III collagen, fibronectin, and the anti-protase α2-macroglobulin (11, 13). Of particular note, a single serotonin receptor

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1 The abbreviations used are: MMP-13, matrix metalloproteinase-13; D609, tricyclodec-an-9-y1-xanthogenate; DAG, diacylglycerol; DOG, 1,2-dioctanoyl-sn-glycerol; DOI, 1-(2,5-dimethoxy-4-isopropyl phenyl)-2-amino propane HCl; ERK, extracellular signal-regulated kinase; 5-HT, 5-hydroxytryptamine; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PI, phosphatidylinositol; IP, inositol phosphate; PC, phosphatidylcholine.

2 J. L. Sanchez and J. J. Jeffrey, unpublished data.

3 J. A. Dumin and J. J. Jeffrey, manuscript in preparation.
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is responsible for mediating both the positive- and negative-regulated serotonin-dependent processes in myometrial smooth muscle cells; the 5-HT_{2A} subtype (14). Although these divergent biochemical processes are mediated through a single receptor subtype, they possess different postreceptor mechanisms. Specifically, while the serotonin-dependent regulated genes, MMP-13, transin, 92 kDa gelatinase, IL-1, and IL-6, appear to require IL-1, the down-regulated genes, types I and III collagen, fibronectin, and α_{2}-macroglobulin, are independent of IL-1 influence. Furthermore, phorbol esters, known activators of protein kinase C (PKC), mimic the effects of serotonin on the serotonin-dependent-induced genes while having no effect on the serotonin-dependent down-regulated genes (13, 15). Conversely, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-bromo-cyclic AMP) mimics the effects of serotonin on the negatively regulated gene products for type I collagen and α_{2}-macroglobulin (11, 13). Thus, the serotonin-mediated induction of MMP-13 and accompanying down-regulation of collagen appears to be via two distinct signaling pathways that must be stringently controlled to preserve the integrity of the uterus during pregnancy.

The 5-HT_{2A} receptor subtype possesses the 7 transmembrane domains characteristic of G-protein-coupled receptors. Binding of serotonin has been shown to increase intracellular Ca^{2+} levels (16, 17) and to stimulate phospholipase C (PLC) for the production of inositol phosphates (18–21) and diacylglycerol (DAG), both activators of PKC. Like other G-protein-coupled receptors, this activation of PKC through the 5-HT_{2A} receptor stimulates the mitogen-activated protein kinase (MAPK) cascade, leading to the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (22–24). Although many postreceptor signaling cascades have been described for the 5-HT_{2A} receptor in other systems, little is known about the signaling pathway(s) involved in serotonin-mediated induction of MMP-13 in rat myometrial SMCs. This study indicates that serotonin-induced MMP-13 protein production requires the sequential activation of a phospholipase C pathway, protein kinase C, and ERK1/2.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—Dulbecco’s Modified Eagle’s Medium (DMEM) and inositol-free DMEM were obtained from Invitrogen. Fetal bovine serum (FBS) was obtained from Hyclone Inc., Logan, UT. DMEM and inositol-free and serum-free DMEM supplemented with 30 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 ng/ml LPS were maintained under these conditions until experimental treatments began.

Serotonin Phosphate Assay—Cells were labeled for 18–24 h with 0.25 μCi/ml [3H]inositol in inositol-free and serum-free DMEM supplemented with 30 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 ng/ml LPS. Immediately prior to experimental treatments, cells were washed with 1–2 ml of inositol-free DMEM. Cells were pretreated in 0.5 ml of the same media containing 20 mM LiCl and, if necessary, the appropriate inhibitors and vehicles for the inhibitors for 30 min before experimental treatments. The cells were then added to challenge the cells (1 h, 37 °C). After the appropriate incubation time had passed, the cells were washed with 2 ml of phosphate-buffered saline (PBS) and aspirated, and 0.75 ml of ice-cold 20 mM formic acid was then added to stop the reaction. The cells were then placed at −20 °C for >1 h.

The formation of [3H]inositol phosphates (IP) in response to serotonin agonist challenge was determined via ion column chromatography. Supernatant fractions were loaded on to AG1-X8 dowex columns, and the total [3H]inositol fraction was eluted with 50 mM ammonium solution (pH > 9.0). The bound [3H]inositol phosphates were eluted with 2 μl ammonium formate. Total IP production was then estimated by determining the ratio of [3H]IP to [3H]inositol plus [3H]IP as described previously (26, 27). Protein Extraction of MMP-13—Primary cultures of myometrial smooth muscle cells upon reaching confluence were incubated with appropriate compounds, including vehicle controls, in inositol-free and serum-free DMEM for varying times dependent upon treatments, and the media was then removed. Each sample of media was incubated overnight at 4 °C with a 50-μl slurry of heparin-Sepharose beads in 0.05 M Tris/0.1 M CaCl₂/0.2 mM NaCl per 1–2 ml of media to preferentially bind the MMP-13 protein. Dextran sulfate (50 μg/ml 0.05 μM Tris, 0.01 mM CaCl₂) was then used to elute the collagenase off the heparin-Sepharose beads.

PKC Isoforms—For protein extraction of PKC isoforms, cells were pretreated for 1 h with the inhibitors and the appropriate vehicle controls, followed by serotonin for 7.5 min, rinsed 2× with ice-cold PBS and scraped off 75 mm² flasks with 700 μl of lysis buffer containing protease inhibitors (5 mM benzamidine, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 5 μg/ml peptatin A, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na₃VO₄, 1 mM para-nitrophenyl phosphate, and 5 mM imidazole). The cells were then sonicated (for 40 s at 50% duty cycle and with pulsation) and spun at 4 °C for 45 min at 55,000 rpm. The cytosolic fraction (supernatant) was retained while the pellet was resuspended in 200 μl of lysis buffer and 1% Triton X-100. The tubes were then vortexed, left on ice for 20 min, and spun at 14,000 rpm in a microcentrifuge at 4 °C for 5 min. The membrane fraction (supernatant) was then resuspended in 2× sample buffer. Protein determination assays were performed with the Pierce BCA protein assay kit or the BioRad Laboratories protein assay kit.

ERK1/2—The ERK1/2 proteins were isolated from cells plated in 75-cm² flasks. Inhibitors and vehicle controls were preincubated for 1 h, and then serotonin was added for 7.5 min, with the exception of the time course studies, and the cells were rinsed 2× with ice-cold PBS and then scraped with 250 μl of PBS. The cells were spun down for 30 min at 14,000 rpm at 4 °C and then lysis buffer containing protease inhibitors (200 μg/ml HEPES, 10 mM KCl, 1 mM EDTA, 10% glycerol, 20 mM NaF, and 1 mM dithiothreitol) containing protease inhibitors (3 μg/ml aprotinin, 0.1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride) and 0.2% Nonidet P-40 for 10 min on ice. The samples were spun at 13,000 rpm for 2 min at 4 °C and the supernatant retained as the cytosolic fraction. The pellet was resuspended in high salt hypotonic buffer (hypotonic buffer as described above with 20% glycerol and 420 mM NaCl) and protease inhibitors and rocked overnight at 4 °C. The next day, the samples were spun at 4 °C for 10 min at 13,000 rpm and the supernatant retained as the nuclear fraction. Protein determination assays were performed as noted above.

Western Blot Analysis—Protein samples were electrophoresed on 12% (α/β) and 10% (α/ERK1/2), and 7.5% (PKC) SDS-polyacrylamide gels under reducing conditions following protocols established by Towbin et al. (28). The proteins were transferred to polyvinylidene difluoride membranes and incubated with the appropriate primary antisera. MMP-13 antisera was used at a 1:2500 dilution. All PKC antibodies were obtained from Transduction Laboratories, Lexington, KY except for PKCε (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PKCγ and PKCζ (BioMol Research Laboratories, Inc., Plymouth Meeting, PA).
A. Effects of 5-HT and DOI on IP Production

B. Effects of 5-HT and 5-HT2A-R Inhibitors on IP Production

C. Effects of DOI and Spiperone on IP Production

D. Effects of U73122 and U73343 on IP Production

E. Effects of U73122 and U73343 on MMP-13 Expression

F. Western Blot for Collagenase

Fig. 1. Effects of serotonin, a 5-HT2A receptor agonist, 5-HT2A receptor antagonists, and the PLC inhibitor U73122 on IP production over time. Cells were plated on 12-well plates and grown to confluence in DMEM supplemented with 10% FBS (v/v), 30 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 ng/ml LPS until day 5. At that time, the cells were then maintained in DMEM supplemented with 10% serotonin-free FBS (CS-FBS) (v/v), 30 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 ng/ml LPS until day 8. Treatments were in inositol-free and serum-free DMEM (supplemented with 30 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, and 100 ng/ml LPS) containing 20 mM LiCl. The reaction was stopped at the appropriate time points with 20 mM formic acid, and the cells were placed at −20 °C for ≥1 h, thawed and total inositol phosphates were measured. A, 5 μM serotonin or the 5-HT2A receptor agonist DOI was added to challenge the cells (37 °C). B, cells were pretreated with the 5-HT2A receptor-specific inhibitors ketanserin (1 μM) or spiperone (1 μM) as indicated for 15–30 min. 5 μM serotonin was then added for the appropriate time points (37 °C). C, cells were pretreated with 1 μM serotonin Induction of MMP-13 through PLC, PKC, and ERK1/2.
PKC isoform antibody dilutions were as follows: PKCα 1:1000, PKCβ 1:250, PKCγ 1:1000, PKCδ 1:500, PKCε 1:1000, PKCι 1:250, PKCλ 1:250, PKCω 1:250, and PKCβ 1:100. ERK1/2 and phospho-ERK1/2 (pERK1/2) antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. ERK1/2 and pERK1/2 antibodies were used at a 1:1000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG were used as the secondary antisera at a 1:10000 dilution. The proteins were detected using a horseradish peroxidase substrate kit from Amersham Biosciences.

RESULTS

Serotonin-induced Up-regulation of MMP-13 Is PLC-dependent

Effects of Serotonin, DOI, and 5-HT2A Receptor Antagonists on Inositol Phosphate Production—Serotonin-mediated MMP-13 production occurs through the 5-HT2A receptor, and this receptor is known to mediate phospholipase C activation (18–21). We have previously demonstrated that 5-HT2A receptor-selective agonists (1-(2,5-dimethoxy-4-iodophenyl)-2aminopropane HCL (DOI) and quipazine) and antagonists (ketanserin and spiperone) can mimic and inhibit, respectively, the effects of serotonin on MMP-13 production (14). To determine if antagonists (ketanserin and spiperone) can mimic and inhibit, respectively, the serotonin-mediated response was blocked in the presence of the 5-HT2A receptor-specific antagonists ketanserin (1 μM) or spiperone (1 μM) (Fig. 1B). The receptor agonist DOI mimicked the effects of serotonin on IP production (Fig. 1A), an effect that was attenuated by spiperone (Fig. 1C). These results indicate that in rat myometrial smooth muscle cells, serotonin can activate PLC through the 5-HT2A receptor subtype.

Effects of the PI-PLC Inhibitor U73122 and Its Inactive Analogue U73343 on Inositol Phosphate Turnover and MMP-13 Protein Expression—To further confirm the role of phospholipase C in the production of MMP-13, the PLC inhibitor U73122 and its inactive analogue U73343 were used to determine their effects on serotonin-induced inositol phosphate turnover. U73122 inhibited the release of inositol phosphates ∼50% while the inactive analogue U73343 had no effect (Fig. 1D). This inhibitory response was dose-dependent with maximal inhibition at 20 μM U73122. At this same concentration, U73122 was able to attenuate the serotonin-dependent production of MMP-13 protein expression following serotonin treatment while U73343 did not exert a significant effect on MMP-13 protein levels (Fig. 1, E and F). The two bands seen in the MMP-13 standard are representative of the pro-form of MMP-13 and its active counterpart, which has approximately a 10-kDa loss in mass.

Effects of the PC-PLC Inhibitor D609 on Inositol Phosphate Turnover and MMP-13 Protein Levels—Initially, we utilized the PC-PLC inhibitor tricyclodecan-9-yl-xanthogenate (D609) as a negative control for our PI-PLC activity assays. Interestingly, measurements of IP generation in the presence of serotonin revealed that D609 depleted inositol phosphate production in a dose-dependent manner (Fig. 2A); the maximal effect was seen at 250 μM. This inhibition maximally decreased IP turnover by ∼50% (Fig. 2, A and B), as seen with U73122. Similarly, MMP-13 protein production was attenuated by D609 and reached levels comparable to those of the negative control by 150 μM D609 (Fig. 2C). Although the cells were viable after D609 treatment, we wanted to determine if there had been some detrimental effects to their cellular functions that might account for the inhibitory effects on IP production. To that end, we examined if the effects of D609 on serotonin-stimulated PLC activity and MMP-13 production were reversible. D609 was incubated overnight and then washed out of the cells prior to treatment with serotonin. Recovery of serotonin-mediated IP production was seen 1-day post-D609 treatment and IP turnover was higher than that of the control (Fig. 2D, left). However, in the days following, the levels of IP production equalized between the cells that had been treated with D609 and controls. With respect to D609 effects on MMP-13 levels, protein production recovered within 6 h of D609 removal and serotonin challenge (Fig. 2D, right). The particular control sample (+5HT) at 6 h with nearly undetectable MMP-13 production was likely due to the occasional differences seen in varying primary cell culture preparations. These results suggest that PC-PLC may regulate IP generation and MMP-13 production in rat uterine SMCs by an as yet unknown mechanism.

Role of PKC in MMP-13 Expression

DOG and PMA Regulate MMP-13 Expression—The second messenger DAG, produced by the activation of phospholipase C, is a known activator of protein kinase C. To examine the role of PKC in MMP-13 production, cells were challenged with DOG, a cell-permeable DAG analogue, for 6 h. DOG was able to induce the production of MMP-13 to a level comparable to that of serotonin (Fig. 3A). Wilcox et al. (15) have shown that short-term exposure to phorbol myristate acetate mimics the effects of serotonin on MMP-13 protein production in myometrial smooth muscle cells. On the other hand, prolonged exposure to PMA is known to deplete PKC (29–31). Cells were pretreated for 24 h with 25 nM PMA to down-regulate phorbol ester-sensitive PKC isoforms and then challenged with either 5 μM serotonin or 25 nM PMA for 6 h. Control cells were not exposed to PMA and were treated in concert with the pretreated cells, and the media collected after 6 h. Acute exposure to PMA stimulates the production of higher levels of MMP-13 protein than that of the serotonin-stimulated response (Fig. 3A). However, cells that had been pretreated with PMA to deplete PKC protein expressed significantly lower levels of MMP-13 protein as compared with cells exposed to either serotonin or PMA for only 6 h. Following down-regulation with PMA, subsequent agonist challenge with either serotonin or PMA did not elicit a comparable response to that of controls. These results support a role for PKC in the regulation of MMP-13 expression in rat myometrial SMCs.

PKC Isoform Expression in the Cytosol of Postpartum Rat Myometrial Smooth Muscle Cells—PKC isoforms α, β, δ, ε, ζ have been identified in the myometrium of pregnant and non-pregnant rats. Since 4-day postpartum uteri are used to obtain our rat primary cultures of myometrial smooth muscle cells, we wanted to determine if there were any significant differences in PKC isoform expression in this cell system. The PKC isoforms that appear to be present in the cytosol of postpartum uteri are

spiperone for 15–30 min, and then 5 μM DOI was added for the appropriate time points (37 °C). Data represent the mean ± S.E. of triplicate determinations (n = 3) (3 wells/treatment/experiment). A–C, *p < 0.05; comparison to control (−5HT). D, cells were pretreated with varying concentrations of the PLC inhibitor U73122 and its inactive analogue U73343 for 1 h prior to serotonin challenge. Data represent the mean ± S.E. of duplicate determinations (n = 2) (3 wells/treatment/experiment). *p < 0.05; comparison to control (+5HT). E and F, Western blot analysis was performed on cells pretreated with U73122 or U73343 for 1 h prior to serotonin challenge. After 6 h, the media was removed and collagenase protein extracted with heparin-Sepharose beads, and equal volumes were run on a 12% SDS-PAGE gel. Data represent the mean ± S.E. of duplicate determinations (n = 2) (3 wells/treatment/experiment).
FIG. 2. Effects of the PLC inhibitor D609 on IP production and MMP-13 protein levels. Cells were grown and maintained as described in the legend to Fig. 1. Cells were pretreated with either varying concentrations of the PLC inhibitor D609 for 1 h (A) or treated with 250 μM D609 for varying time points prior to serotonin challenge (B). Data represent the mean ± S.E. of duplicate determinations (n = 2) (3 wells/treatment/
Fig. 3. Effects of PKC activation on MMP-13 expression and the effects of serotonin on PKC. Cells were grown and maintained as described in the legend to Fig. 1. A, DOG, a DAG analogue, was added for 6 h and MMP-13 protein extracted as described under “Experimental Procedures.” Cells were pretreated with 25 nM PMA for 24 h prior to serotonin/PMA challenge for an additional 6 h. Control cells were not exposed to a pre-treatment of PMA. MMP-13 protein was collected and Western blot analysis performed as previously described (n = 2). B, PKC protein was isolated as described under “Experimental Procedures.” 400 μg of total protein of cell lysates was run on a 7.5% SDS-PAGE gel for Western blot analysis and antibody incubations (PKC-MW kDa: α77, β77, γ78, δ78, ε83, λ74, ν74, π78, and ε88) were performed in a Hoeffer PR 150 Deca-Probe Incubation Manifold (n = 2). C, protein fractions of cell lysates were run on a 7.5% SDS-PAGE gel for Western blot analysis (n = 2). Numbers in the table are densitometry readings that correspond to percent change from control ± S.E. N.D., not determined.

α, β, δ, ε, λ/ε, η, and ζ (Fig. 3B). These isofoms represent all three classes of PKC, classical (α, β, εPKC), novel (δ, ε, nPKC) and atypical (λ/ε, ζ, ωPKC). The PKC isoforms λ and δ appear to be the most highly expressed within the rat myometrium while the γ isoform was undetectable.

Protein Kinase C-α, −β, −δ, −ε, and −ζ Isoform Expression in Cytosol and Membrane Fractions following Serotonin Treatment—The translocation of PKC isoforms from the cytosol to the membrane is considered to be a determinant of PKC activation (32). To identify which PKC isoforms in particular participate in the signaling cascade initiated by serotonin, which results in MMP-13 production, we examined the expression of various PKC isoforms in both the cytosol and membrane fractions of cell lysates following serotonin treatment. Upon the addition of serotonin, there was a marked decrease (~21%) in PKCδ expression in the cytosolic fraction of cell lysates and a corresponding increase (~32%) in the membrane fraction (Fig. 3C). PKCε also showed a similar expression pattern following serotonin treatment with increased translocation to the membrane (+36%). On the other hand, PKCα expression was present in the cytosolic fractions of all the experimental treatments with an increase in expression after exposure to serotonin. However, PKCα expression was nearly undetectable in the membrane fractions of the cell lysates of rat myometrial smooth muscle cells. Neither PKCβ nor PKCζ exhibited translocation from the cytosol to the membrane following serotonin treatment. These results are consistent with a role for the nPKC isoforms δ and/or ε in the signaling pathway from PLC to MMP-13 protein production.

Effects of the Protein Kinase C Inhibitors Bisindolylmaleimide I, CGP 41251, and Calphostin C on MMP-13 Protein Production—The PKC activator DOG up-regulated the production of MMP-13 in rat myometrial smooth muscle cells. Activation of PKC by acute exposure to PMA, and the depletion of PKC by prolonged PMA exposure resulted in the up-regulation and down-regulation, respectively, of MMP-13 protein levels. These factors then led to further examination of the role of PKC in this system. Bisindolylmaleimide I and CGP 41251, two potent...
Staurosporine analogues that act as competitive inhibitors on the ATP-binding site of PKC and are more selective for the cPKCs, were able to inhibit the serotonin-mediated signal transduction pathway and attenuate the MMP-13 protein production at a concentration of 10 nM (Fig. 4, A and B). Calphostin C, a competitive inhibitor of the diacylglycerol-binding site on the regulatory domain of PKC, was also able to significantly inhibit the production of MMP-13 protein at a concentration of 10 nM (Fig. 4C). These results strongly suggest that a DAG-activated PKC is necessary for serotonin-mediated MMP-13 up-regulation in rat uterine SMCs.

Effects of the Protein Kinase C-δ Isoform-selective Inhibitor Rottlerin on MMP-13 Protein Levels—Due to the fact that the PKC isoform δ is one of the highly expressed isoforms in the postpartum rat uterus, rottlerin, a PKCδ-selective inhibitor, was used to examine the role of that isoform in serotonin-mediated signaling. Rottlerin was able to attenuate serotonin-induced MMP-13 protein production in a dose-dependent manner (Fig. 5). At a concentration of 500 nM, rottlerin was able to inhibit MMP-13 protein accumulation to levels similar to that of the negative control. These data, in concert with those in Fig. 3, provide evidence for PKC, possibly the δ isoform, in regulating the signaling cascade initiated by serotonin in the production of MMP-13.

Serotonin-mediated ERK Phosphorylation and Effects of the MEK Inhibitor PD98059—PKC has been shown to activate, coupled to the 5-HT2A receptor, the MAP kinase signaling pathway of which ERK1/2 is a downstream effector (22–24). Dual phosphorylation of ERK1/2 by MEK has been correlated to the activation of ERK1/2 (33–35). To determine whether ERK1/2 activity is stimulated by serotonin, ERK1/2 phosphorylation over time was measured. ERK1/2 phosphorylation was detected 5 min after exposure to serotonin with maximal phosphorylation achieved within 10 min (Fig. 6A) within both the cytosolic and nuclear fractions. ERK1/2 phosphorylation then diminished at 15 min and returned to basal levels within 30 min following serotonin treatment. This result indicates that serotonin links to the MAP kinase cascade, resulting in ERK1/2 activation.

Having determined that serotonin induced ERK activation, we wanted to determine if ERK phosphorylation was required for MMP-13 protein production. If ERK phosphorylation is necessary for MMP-13 expression, blocking the activation of ERK should in turn attenuate the effects of serotonin in the up-regulation of MMP-13. We found that the MEK inhibitor PD98059, with the inhibition of ERK1/2, was able to suppress MMP-13 protein expression at a concentration of 25 μM (Fig. 6B). Furthermore, PD98059 also abrogated the effects of sero-

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**FIG. 4.** Effects of PKC inhibitors bisindolylmaleimide I, CGP 41251, and calphostin C on interstitial collagenase protein levels. The cells were prepared as described in the text and plated on 12-well plates. The cells were pretreated with varying concentrations of bisindolylmaleimide I (A), CGP 41251 (B), or calphostin C (light-activated) (C) for 60 min prior to serotonin challenge. After 6 h, the media was collected, and interstitial collagenase protein was extracted with heparin-Sepharose beads and equal volumes were run on a 12% SDS-PAGE gel. Data represent the mean ± S.E. of quadruplicate determinations (n = 4) (A, B), or duplicate determinations (n = 2) (C) (3 wells/treatment/experiment). A–C, Δ and *, p < 0.05; Δ is comparison to the control, −5HT and * is comparison to the control, +5HT.
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Effects of Rottlerin on MMP-13 Levels

FIG. 5. Effects of the PKCα-selective inhibitor rottlerin on interstitial collagenase protein production. The cells were pretreated with varying concentrations of rottlerin for 1 h prior to serotonin challenge. After 6 h, the media was collected and interstitial collagenase protein was extracted with heparin-Sepharose beads, and equal volumes were run on a 12% SDS-PAGE gel. Data represent the mean ± S.E. of quintuple determinations (n = 5) (3 wells/treatment/experiment). ∆ and *, p < 0.05; ∆ is comparison to the control (−5HT) and * is comparison to the control, +5HT.

In identifying which PKC isoforms are present in primary cultures of 4-day postpartum rat myometrial SMCs, we found members from all three classes: classical (α, β), novel (δ, ε), and atypical (ξ, η, ζ). Of these three PKC subtypes, only the classical (18, 20, 21). Previous studies from this laboratory have demonstrated that the 5-HT2A receptor is the only serotonin receptor subtype mRNA detectable in rat uterine smooth muscle cells (14). Furthermore, this is the receptor that differentially regulates expression of a number of genes. Serotonin up-regulated genes include IL-1 (12), IL-6,3 transin, and 92 kDa gelatinase,2 while types I and III collagen, fibronectin, and αv-macroglobulin are down-regulated by serotonin (11, 13). While our previous studies have focused on the transcriptional regulation and enzymatic activity of MMP-13 (11, 15, 37), here we have extended those observations to the level of immunoreactive protein. The results of this study further clarify the signaling mechanisms by which serotonin, acting through the 5-HT2A receptor, mediate the production of MMP-13 and demonstrate that phospholipase C, its downstream effector PKC and the MAP kinase cascade are integral components in this cell system.

Our present results indicate that PLC activation is required for MMP-13 protein expression. As illustrated in Fig. 1, serotonin was able to markedly increase IP production specifically through the 5-HT2A receptor subtype. Selective and well-characterized 5-HT2A receptor agonists (38, 39) were able to mimic the effects of serotonin on IP production and conversely, 5-HT2A receptor selective antagonists (40, 41) abrogated the effects of serotonin on IP turnover. Consistent with the hypothesis that PLC is a crucial element of this signaling pathway, the PLC-selective inhibitor U73122 abolished the increased IP turnover and decreased the production of MMP-13 protein. Surprisingly, we found that the putative phosphatidylincholine-specific PLC inhibitor D609 (42, 43) significantly and dose-dependently exerted inhibitory effects upon both the phosphatidylinositol-specific PLC pathway and MMP-13 production in a reversible manner, as shown in Fig. 2. There have been recent reports in which D609 was found to not only exert effects upon PC-PLC but also PLD (44, 45) and PI-PLC (46, 47). These results do not preclude the possible role of PC-PLC or PLD in MMP-13 protein expression. However, our results can serve to further emphasize the role of DAG in regulating MMP-13 expression since DAG is a major product of both PLC, whether it is derived from PI- or PC-PLC, and PLD activity. Furthermore, a DAG analogue alone was able to up-regulate MMP-13 protein expression to levels comparable to that of serotonin.

Following a logical progression from PLC to the activation of PKC by DAG and previous studies where PMA was found to stimulate MMP-13 expression in human (48) and rabbit (49) fibroblasts, as well as rat myometrial smooth muscle cells (15), we examined the possible role of PKC in mediating serotonin signaling. Although expression of PKC isoforms following prolonged PMA exposure were not examined in this study, phorbol ester-induced down-regulation of PKC isoforms has previously been demonstrated in both the human and rat myometrium (50, 51). In our rat myometrial smooth muscle cell system, exposure to PMA for 24 h was sufficient to attenuate the production of MMP-13 protein. Furthermore, the PKC-selective inhibitors bisindolylmaleimide I, CGP 41251, and calphostin C, which have different sites of action upon PKC, were all able to abrogate the effects of serotonin on MMP-13 protein level. These inhibitors exert their effects on either the ATP-binding site (bisindolylmaleimide I, CGP 41251) or the DAG-binding site of PKC (calphostin C), respectively, and provided further evidence that serotonin-mediated MMP-13 production follows a traditional signaling pathway from PLC to PKC.

The purpose of these studies was to identify and order the major players in the signal transduction pathway that is initiated by serotonin binding to the 5-HT2A receptor and results in the production of MMP-13. The 5-HT2A receptor is a G-protein-coupled receptor (36) that has been linked to PLC in a variety of cell systems, including the rat cerebrum, aorta, and uterus.
and novel isoforms possess a DAG requirement. Both DAG analogues, the phorbol ester PMA and DOG, were able to mimic the effects of serotonin on MMP-13 production. This indicated a likely role for one or more DAG-regulated PKC isoform(s) and, taking advantage of the PKCδ/H9254-selective inhibitor rottlerin, we focused on the highly expressed cPKC isoform/H9254. As seen in Fig. 5, rottlerin significantly inhibited MMP-13 protein production. To determine if other PKC isoforms participated in the signaling pathway, we examined PKC isoform translocation, as a measure of activity, from the cytosol to the membrane upon serotonin treatment.

Expression of both the δ and ε isoform diminished within the cytosol while showing a corresponding increase within the membrane fraction. These studies are consistent with a PKC requirement for MMP-13 expression and implicate the PKCδ isoform in this signal transduction pathway. Although neither of the two classical PKC isoforms, α or β, showed detectable translocation to the membrane following serotonin treatment, we cannot dismiss the possibility that they may indeed contribute to the overall induction of MMP-13 since bisindolylmaleimide I and CGBP 41251 are selective for the classical PKC isoforms. There may also be a role for PKCe in this signaling mechanism as evidenced by its increased expression at the membrane following serotonin treatment. Despite the expression of the atypical PKC isoforms in this cell system, it seems unlikely that they influence serotonin-mediated MMP-13 production in light of the potent inhibitory effects of calphostin C, a competitive inhibitor for the DAG-binding site.

The activation of the MAP kinase cascade by PKC has been shown to be involved in 5-HT2A receptor subtype signaling events (22–24). Furthermore, the PKCδ isoform has been...
shown in a variety of other cell types, mainly epithelial in origin but not in myometrial SMCs, to activate the ERK pathway (52–54). Ueda et al. (55) were able to demonstrate this same effect in COS1 cells transfected with constitutively active PKCδ. Previous studies have shown that increased MMP-13 mRNA expression in chondrocytes (56, 57), osteoblasts (58), and synoviocytes (59) are dependent upon ERK1/2 activation. In contrast, other studies have shown that activated ERK1/2 inhibited MMP-13 expression in human dermal fibroblasts (60), and the inhibition of ERK1/2 activity in transformed keratinocytes had no effect upon MMP-13 expression (61). Our studies found that ERK1/2 activation occurs rapidly and subsequently diminishes by 30 min. Utilizing the MEK inhibitor PD98059, we were able to block both ERK phosphorylation and MMP-13 protein production at the same concentrations. This demonstrates that not only does the MAP kinase cascade occur as a result of serotonin treatment but also that ERK phosphorylation is necessary for MMP-13 protein production. These disparate data between cellular systems may be, in part, due to the distinct functions that MMP-13 has in mammalian physiology. The nature of uterine biology and the drive to return to reproductive competence may necessitate the divergent signaling regulation from that seen in wound healing and other physiological processes. The similarities seen in signaling mechanisms associated with MMP-13 expression in uterine remodeling and the pathology of arthritis may perhaps be correlated with the critical role of cytokines in both these processes (12, 62).

To better delineate the relationship between PKC and ERK1/2, the PKC inhibitors rottlerin and calphostin C were used to examine their effects on ERK phosphorylation. Both ERK1/2, the PKC inhibitors rottlerin and calphostin C were used to examine their effects on ERK phosphorylation. Both inhibitors were indeed able to attenuate ERK1/2 activation by inhibitors that PKC activation precedes that of ERK1/2 and that the MAP kinase cascade has an integral part in the overall maintenance of uterine integrity. Activated ERK1/2 levels were increased in both the cytosolic and nuclear fractions following serotonin treatment. While phosphorylated nuclear ERK1/2 may modulate gene transcription, there may be other possible functions for cytoplasmic ERK1/2 in MMP-13 expression and secretion. Liu et al. (63) demonstrated that intracellular levels of MMP-9 and MMP-2 did not parallel the suppression of MMP-9/MMP-2 secretion by dominant negative Ras and thus, indicated that there may be a post-transcriptional mechanism for ERK1/2 regulation of MMP secretion. In terms of what may be occurring downstream of ERK1/2 activation, recent studies from our laboratory have identified the AP-1 site on the rat MMP-13 promoter, along with the extended palindromic sequence that surrounds it, as a requirement for MMP-13 transcription (64). Although the AP-1 site is necessary, it is not sufficient for MMP-13 production.

The role that serotonin-dependent IL-1 expression plays in the above signaling pathway has not been addressed in the present study. Liacini et al. (56) have recently shown that the IL-1-induced MMP-13 expression in articular chondrocytes can be reduced by inhibitors to ERK1/2. Interestingly, Mengshol et al. (65) found that IL-1 induction of MMP-13 in chondrocytes was almost completely abolished by the p38 inhibitor SB203580 whereas the MEK inhibitor PD98059 had no effect on IL-1-dependent MMP-13 protein expression. It may be that IL-1 mediates the phosphorylation of AP-1 binding proteins through p38, which then bind to the AP-1 site in response to serotonin, while serotonin mediates its responses via ERK1/2. Indeed, IL-1 gene expression was demonstrated to be more dependent upon the activation of p38 than of ERK1/2 (66). Another possibility is suggested by recent studies from Reunanen et al. (67) where they found that p38 activation not only induces MMP-1 (collagenase-1) expression but also stabilizes the mRNA by ∼15-fold. Furthermore, an earlier report demonstrated ERK1/2 activation alone potently up-regulates MMP-1 mRNA levels whereas p38 activation has no effect on the MMP-1 promoter (68). IL-1 itself has been shown to stabilize human collagenase mRNA (69). It is likely that IL-1 modulates MMP-13 production in an autocrine manner that augments the effects of serotonin and thus, amplifies the signaling cascade in myometrial SMCs.

Little is known about other possible post-transcriptional mechanisms that may regulate MMP-13 expression and secretion in myometrial smooth muscle cells. With respect to other MMPs, MMP-9 secretion can be inhibited independently of alterations of mRNA levels in cells expressing a membrane-anchored glycoprotein that possesses protease inhibitor-like domains (70). Regulation of MMP-9 secretion in carcinoma cells was determined to be dependent upon the efficiency of translation (71). It is evident by the complex nature of the signaling pathways involved that there may be manifold points of regulation by these effector molecules.

In summary, our overall model (Fig. 7) proposes that serotonin initiates a signal transduction cascade that is mediated by the 5-HT2A receptor subtype in myometrial smooth muscle cells (14) and causes a subsequent activation of PLC through a G-protein to produce DAG. The resultant DAG in turn activates PKC, where the δ isomorph is one likely participant, and PKC then propagates this cascade through ERK1/2 activation, which results in the production of MMP-13. These results demonstrate the multiple regulatory factors required for the stringent control of MMP-13 production in the context of ensuring a timely initiation of postpartum events resulting in the degradation of collagen, as such, may logically contribute to the prevention of inappropriate collagenolysis in the uterus, and the subsequent return of the uterus to reproductive competence.

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REFERENCES
1. Burgese, R. E. (1988) Annu. Rev. Cell Biol. 4, 551–577
2. Harkness, M. L. R., and Harkness, R. D. (1954) J. Physiol. 129, 492–500
3. Harkness, R. D., and Moralée, B. E. (1956) J. Physiol. 132, 502–508
4. Jeffrey, J. J., and Gross, J. (1970) Biochemistry 9, 268–273
5. Strenchik, G. P., Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z. (1977) Biochemistry 167, 1017–1015
6. Blair, H. C., Teitelbaum, S. L., Ehlich, L. S., and Jeffrey, J. J. (1986) J. Cell. Physiol. 129, 111–123
7. Ross, R., and Knehanoff, S. J. (1971) J. Cell Biol. 50, 159–171
8. Roswit, W. T., Rias, L., Gast, M. J., Welgus, H. G., and Jeffrey, J. J. (1988) Arch Biochem. Biophys. 262, 67–75
9. Halme, J., Tyree, B., and Jeffrey, J. J. (1986) Arch Biochem. Biophys. 199, 51–60
10. Jeffrey, J. J., Ehlich, L. S., and Roswit, W. T. (1991) J. Cell. Physiol. 146, 399–406
11. Passaretti, T. V., Wilcox, B. D., and Jeffrey, J. J. (1996) Mol. Endocrinol. 10, 125–132
12. Wilcox, B. D., Dumin, J. A., and Jeffrey, J. J. (1994) J. Biol. Chem. 269, 20655–20664
13. Huang, C., and Jeffrey, J. J. (1998) Mol. Cell. Endocrinol. 139, 79–87
14. Rydelek-Fitzgerald, L., Wilcox, B. D., Teitler, M., and Jeffrey, J. J. (1993) Mol. Cell. Endocrinol. 91, 67–74
15. Wilcox, B. D., Rydelek-Fitzgerald, L., and Jeffrey, J. J. (1992) J. Biol. Chem. 267, 20762–20767
16. Nakaki, T., Roth, B. L., Chuang, D. M., and Costa, E. (1985) J. Pharmacol. Exp. Ther. 234, 443–446
17. Watts, S. W., Cox, D. A., Johnson, B. G., Schoepp, D. D., and Cohen, M. L. (1994) J. Pharmacol. Exp. Ther. 271, 832–844
18. Conn, P. J., and Sanders-Bush, E. (1984) Neuropharmacology 23, 993–996
19. Conn, P. J., and Sanders-Bush, E. (1985) J. Pharmacol. Exp. Ther. 234, 195–203
20. Roth, B. L., Nakaki, T., Chuang, D. M., and Costa, E. (1986) J. Pharmacol. Exp. Ther. 238, 480–485
21. Cohen, M. L., and Wittenauer, L. A. (1987) J. Cardiovasc. Pharmacol. 10, 176–181
22. Greene, E. L., Houghton, O., Collinsworth, G., Garnovskaya, M. N., Nagai, T.,
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