ERK2 mediates oxytocin-stimulated PGE$_2$ synthesis

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Strakova, Zuzana, John A. Copland, Stephen J. Lolait, and Melvyn S. Soloff. 1998. — Oxytocin (OT) induces PG synthesis by both uterine endometrial and amnion cells. We showed previously that CHO cells stably transfected with the rat oxytocin receptor (CHO-OTR cells) also synthesize PGE$_2$ in response to OT. In the present work we have demonstrated that OTRs are coupled to both G$_i$ and G$_q$, using immunoprecipitation of solubilized OTR complexes and ADP ribosylation. OT treatment caused the rapid phosphorylation of extracellular signal-regulated protein kinase 2 (ERK2 or p42MAPK), which was partially inhibited by pertussis toxin (PTX), consistent with OTR-Gi coupling. The PTX-insensitive portion of ERK2 phosphorylation was linked to G$_q$, as inhibitors of both phospholipase C (U-73122) and protein kinase C (GF-109203X) blocked OT-induced ERK2 phosphorylation. OT-stimulated c-fos expression was also mediated by ERK2 phosphorylation. The ERK-c-fos pathway has been shown to be associated with cell proliferation, but OT had no effect on $[^3]$H]thymidine uptake by CHO-OTR cells. However, inhibition of OT-induced ERK2 phosphorylation with an ERK kinase inhibitor (PD-98059) markedly reduced OT-stimulated PGE$_2$ synthesis, pointing to the importance of ERK2 activation in OT action.

Mitogen-activated protein kinases (MAPKs) have been shown to be vital signal pathway components in an increasing number of hormonally responsive cells. These enzymes function as integrators of mitogenic and other signals originating from distinct classes of cell surface receptors, such as tyrosine kinase and G protein-coupled receptors (31). Three subgroups of the MAP kinase family have been identified and cloned: extracellular signal-regulated protein kinase (ERK), stress-activated protein kinase, or c-jun NH$_2$-terminal kinase, and p38 MAP kinase (p38MAPK). These kinases are structurally related, dually phosphorylated on tyrosine and threonine residues, and activated by upstream kinases (11). The various members of the MAP kinase families differ in their substrate specificity, and they are also activated by distinct upstream regulators and extracellular stimuli. In their activated forms, ERK1 and ERK2 transmit extracellular stimuli by phosphorylating a variety of substrates, including transcriptional factors and other kinases (6). ERKs also mediate transcriptional activation of immediate early genes such as c-fos and c-jun.

The pathways originating from many G protein-coupled receptors in the activation of MAPKs are still being defined, and very little is known about the role of MAPK in OT action. Ohmichi and co-workers (24) showed that OT activated p42MAPK (ERK2) phosphorylation and activity in human myometrial cells. The major effect of OT on myometrial cells is activation of contraction, but the effects of OT stimulation of p42MAPK were not reported. Several studies have indicated that ERKs phosphorylate and thereby activate cytoplasmic phospholipase A$_2$ (cPLA$_2$) to produce arachidonic acid, the substrate for PG synthesis (21, 26). These findings are not general, however, because PG synthesis in other cell types is ERK independent (7). One of the aims of

IN RODENTS AND OTHER ANIMALS pregnancy terminates after luteolysis, which occurs naturally at term (29) or can be induced experimentally with PGF$_2$$\alpha$ (4). Recent work with PGF$_2$$\alpha$ receptor knockout mice illustrates the critical relationship between PGF$_2$$\alpha$, progesterone, and the initiation of parturition (29). These mice appear normal except for their inability to deliver pups at term. Blood progesterone levels, which normally decline before the end of term, remain elevated, and the upregulation of oxytocin (OT) receptor (OTR) mRNA in the myometrium that normally occurs immediately before term is lacking. The knock-out phenotype could be reversed by ovariectomy on day 19 of pregnancy, which causes a sharp fall in serum progesterone, induction of OTR mRNA, and parturition. It is thought that OT is the signal for PGF$_2$$\alpha$ release from the decidua in the rat (8) and other species. OT-induced luteolysis leads to the upregulation of myometrial OTR, which sensitizes the uterus to basal levels of OT in the blood, resulting in the initiation of labor contractions (3).

OT action, which is mediated by G$_q$, stimulates phosphoinositol-specific phospholipase C (PI-PLC), leading to increased hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) (17, 22). Both IP$_3$ and DAG play important roles as intracellular second messengers that increase intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]) and activate protein kinase C (PKC), respectively. To better understand the signaling pathways involved in OT-stimulated synthesis of PGs, we stably transfected CHO cells with the rat OTR (CHO-OTR cells) and showed that OT elicited PGE$_2$ synthesis (20). OT treatment also caused a rapid increase in [Ca$^{2+}$], and increased production of inositol phosphates and arachidonic acid release (20). Because these responses to OT are the same as those in primary cultures of endometrial tissue (17) and amnion (22) cells, CHO-OTR cells are useful for studying signal pathways in OT-induced PGE$_2$ synthesis. A distinct advantage of CHO-OTR cells over primary cultures is their uniformity and ability to give reproducible results.

Mitogen-activated protein kinases (MAPKs) have been shown to be vital signal pathway components in an increasing number of hormonally responsive cells. These enzymes function as integrators of mitogenic and other signals originating from distinct classes of cell surface receptors, such as tyrosine kinase and G protein-coupled receptors (31). Three subgroups of the MAP kinase family have been identified and cloned: extracellular signal-regulated protein kinase (ERK), stress-activated protein kinase, or c-jun NH$_2$-terminal kinase, and p38 MAP kinase (p38MAPK). These kinases are structurally related, dually phosphorylated on tyrosine and threonine residues, and activated by upstream kinases (11). The various members of the MAP kinase families differ in their substrate specificity, and they are also activated by distinct upstream regulators and extracellular stimuli. In their activated forms, ERK1 and ERK2 transmit extracellular stimuli by phosphorylating a variety of substrates, including transcriptional factors and other kinases (6). ERKs also mediate transcriptional activation of immediate early genes such as c-fos and c-jun.

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the present studies was therefore to clarify the role of ERK in OT-stimulated PGE2 synthesis in CHO-OTR cells. Recently, we showed that OTRs in the rat myometrium are coupled to Gs, as well as Gq/11 (28). Several G protein-coupled receptors phosphorylate Raf-1 by a tyrosine kinase/Ras-dependent activation through the βγ-subunits of Gs, independent of PKC (31). This pathway has been shown to function with acetylcholine muscarinic receptors (13), lysophosphatidic acid receptors (18, 32), α2-adrenergic receptors (1), and thrombin receptors (32). There is also strong evidence from work with several G protein receptors that Gq/11-mediated receptors (32). There is also strong evidence from work with several G protein receptors that Gq/11-mediated PKA activity stimulates MAPK kinase (Raf-1) by a Ras-independent pathway (31). A third possible mode of MAPK activation is through increases in intracellular Ca2+, which can result in phosphorylation of P38. Phosphorylated Pyk2 then forms a complex with activated Src and Grb2-Sos in the Ras/MAPK signaling pathway (14). Eguchi and co-workers (15) have also shown that the activation of MAPK by angiogenulin II in cultured rat vascular smooth muscle cells is PKC-independent, and possibly involves Gq-mediated p21ras activation via a Ca2+-calmodulin-sensitive tyrosine kinase. The present studies clarify the contributions of Gs and Gq to ERK phosphorylation in OT-stimulated CHO-OTR cells. In addition, we have examined the role of intracellular Ca2+ in OT activation of ERK phosphorylation. We found that the principal pathway involved in OT stimulation of PGE2 synthesis was Gq-PKC-mediated ERK2 phosphorylation.

MATERIALS AND METHODS

Reagents. Reagents were obtained from the following sources: OT and OT antagonist (OTA) (di-His2, Tyr(Me)2, Thr3, Tyr-NH2)γ-jornithine vasotocin, Peninsula Laboratories (Belmont, CA); pertussis toxin (PTX), U-73122, 1,2-bis(2-aminophenoxy)ethane-N,N,N,N′,N′,N′-tetraacetic acid-acetoxyethyl ester (BAPTA-AM), 3,4,5-trimethoxybenzoic acid 8-(2-aminoethyl)octyl ester (TMB-8)·HCl, antibody A-23187 (Calcimycin), GF-109203X, Biomol Research Laboratories (Plymouth Meeting, PA); PD-98059, New England Biolabs (Beverly, MA); [methyl-3H]thymidine (25 Ci/mmol) and Na-125I (Amersham Life Science (Arlington Heights, IL)); [α-32P]dCTP, 3,000 Ci/mmol, [γ-32P]ATP, 3,000 Ci/mmol, NEN (Boston, MA); antibodies specific for ERK2/1 (C-14), Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines. Chinese hamster ovary cells (CHO-K1, ATCC CCL61) were maintained in α-MEM (GIBCO BRL, Grand Island, NY) containing 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. CHO-K1 cells stably transfected with cDNA for rat OTR (CHO-OTR) (20) were cultured as above, with the addition of Geneticon (GIBCO), 400 µg/ml. All cells were maintained at 37°C, under an atmosphere of 5% CO2.

Transfection of CHO-OTR cells, preparation of membranes, solubilization of occupied receptor, size exclusion chromatography, immunoadsorption with G protein α-subunit antibodies, inhibition of PTX catalyzed ADP-ribosylation by OT. These procedures were carried out as described previously (28). Membranes isolated from CHO-OTR cells were solubilized after incubation with [125I]OTA. Solubilized proteins were separated by gel filtration on a fine-performance liquid chromatography Superose 12 column into two major [125I]-OTA-binding fractions: 1) a macromolecular complex (∼400 kDa) and 2) a peak, ∼50–60 kDa, corresponding in size to the OTR. The 400-kDa fraction, containing OTR complexed to signal transduction components, was incubated with specific anti-G protein antibodies (10 µg) or preimmune IgG (PI) as a control. Samples were then assayed to protein A-Sepharose columns, and the amount of radioactivity remaining on the columns after rinsing was determined. The percent of counts per minute bound is expressed relative to the total radioactivity applied to each column. To demonstrate specificity, 10 µg of peptide hapten were coincubated with membrane extracts and antibodies.

Preparation of cell lysates. Cells were grown to confluence on 35-mm (diameter) dishes and maintained in serum-free medium for 18 h. After different drug treatments, the cells were rinsed twice with ice-cold PBS, pH 7.4, and lysed on ice with 150 µl of lysis buffer (25 mM Tris·HCl, pH 7.5, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 mM okadaic acid, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1% Triton X-100, and 0.1% SDS). The extracts were centrifuged at 12,000 g for 20 min at 4°C, and the supernatant fractions were used either to measure kinase activity or frozen at −20°C for subsequent immunoblotting.

Immunoblotting. Cell lysates were applied to 10% SDS-PAGE and were transferred into polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in PBS containing 3% BSA and 0.1% Tween 20. Incubation with primary antibody (see legend in Figs. 1–9 for specific antibodies) for 45 min and secondary IgG antibody for 1 h. Immunocomplexes were visualized by enhanced chemiluminescence (Amersham). All operations were carried out at room temperature. Blots were densitometrically scanned and analyzed using a Dektaste III scanner and PDI 1D software package (PDI, Hunting Station, NY). Quantification of pp42 (phosphorylated ERK2) was carried out by expressing its absorbance relative to the total absorbance (integrated area) of both pp42 and p42 bands. Maximal OT stimulation usually resulted in phosphorylation of 50–60% of total ERK2.

MAP kinase in vitro activity assay. Cell lysates (5 µg of protein in 5 µl) were incubated with 40 µl of kinase buffer (25 mM Tris·HCl, pH 7.4, 10 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 0.5 mM EGTA) at 30°C for 10 min (18). The reaction was stopped by adding 70 µl of Laemmli sample preparation solution. Samples were separated on 15% minigels and transferred upon PVDF membranes, and radioactivity was quantified using a PhosphorImager Scanner (Molecular Dynamics) and ImageQuant program. Autoradiography was carried out by exposure of the gels to BIOMAX MS film (Kodak).

3H]Thymidine incorporation. CHO-OTR cells (10,000/well) were seeded into wells of 12-well plates and grown for 4 days until confluent. The cells were then incubated for 16 h with α-MEM and antibiotics alone (basal) or with either 50 nM OT, 5% FBS, or basic fibroblast growth factor (bFGF), 100 ng/ml. [Methyl-3H]Thymidine (1 µCi) and thymidine (3 µM) were then added to each well, and the incubation was continued for another 4 h. Cells were rinsed with ice-cold PBS twice and were treated with ice-cold 10% TCA. The cells were then rinsed twice with PBS and solubilized in 400 µl of a solution composed of 0.03% SDS in 0.3 N NaOH. The extracts were neutralized with HCl, and radioactivity incorporated into the cells was quantified by liquid scintillation spectrometry.

Northern blot analysis. RNA was isolated from cells using the method of Chomczynski and Sacchi (9). The poly(A) RNA fraction was prepared using the PolyATtract mRNA Isolation System (Promega, Madison, WI) and fractionated. Poly(A)
RNA (400 ng) was fractionated, using denaturing MOPS-formaldehyde-1% agarose gels, transferred to Biotrans nylon membranes (ICN, Irvine, CA) by blotting, and fixed by baking the filters at 80°C for 2 h under vacuum. Human c-fos (33) and chicken β-actin probes (10), both containing the entire coding region, were labeled by random priming with [α-32P]dCTP, using the Megaprime DNA labeling system (Amersham). After hybridization, the nylon membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C. The membranes were then stripped of the c-fos probe by incubation in buffer containing 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 0.5% (wt/vol) SDS for 15 min at 80°C. The membranes were then rehybridized with the β-actin probe and exposed again to X-ray film. The intensity of the c-fos and β-actin bands was determined by densitometry (see above), and the signal intensity of c-fos mRNA was expressed relative to that of β-actin mRNA.

PNG assay. The PNG concentration in cell culture medium was estimated using a PNG enzyme immunoassay kit from Amersham Life Sciences (Buckinghamshire, UK). The sensitivity of assay was 2.5 pg/ml of medium.

Statistical analysis. One-factor ANOVA was used to test the overall hypothesis of no group differences, followed by two-sample t-tests for pairwise comparisons where the overall hypothesis was rejected. All tests were made at the 0.05 level of significance.

RESULTS

Validation of Gα-OTR coupling in CHO-OTR cells. Because the βγ-subunits of Ga were major activators of MAPK in some cell types, it was important to determine whether OTRs are coupled to Ga in CHO-OTR cells. Ga coupling was established by two independent approaches. Physical association of Ga with the OTR was shown by coprecipitation experiments, using antibody to Ga3 α-subunit and 125I-OTA-labeled OTR (Fig. 1A). As in the case of rat uterine myometrial membranes, both Gaq11 and Ga were associated with the OTR in approximately equal amounts (28). The addition of PTX to CHO-OTR membranes without OT caused ADP ribosylation of 41 kDa Ga3, as demonstrated by SDS-PAGE and autoradiography (Fig. 1B). Ribosylation was inhibited by increasing concentrations of OT (Fig. 1B), due to OT-induced dissociation of Ga3 from the heterotrimeric Ga protein complex. Free Ga3 is a substrate for PTX-stimulated ADP ribosylation. The addition of 10 nM OT resulted in about a 50% decrease in the ADP ribosylated α-subunit of Ga3. These results show that the CHO-OTR cell line is a valid system in which to study both PTX-dependent and -independent regulation of MAPK activity.

OT-stimulated ERK2 phosphorylation. CHO-OTR cells were treated with OT for increasing times, and cell lysates were analyzed for phosphorylation of ERK1 and ERK2. OT caused the rapid phosphorylation of ERK2, as evidenced by the electrophoretic mobility shift of a fraction of total ERK2 on immunoblots (Fig. 2A). Phosphorylation gel shifts were detected with antibody cross-reacting to both ERKs, but ERK1 was not phosphorylated after OT addition. The level of phosphorylation of ERK2 peaked around 2 min and returned to basal values by 15 min (Fig. 2A). Comparable results were obtained by assaying ERK activity, which results in the phosphorylation of MBP in vitro (Fig. 2B). MBP phosphorylation was increased about ninefold by 3 min after OT treatment. OT had no effect on ERK2 phosphorylation in CHO cells lacking the OTR (Fig. 3A). Treatment of CHO-OTR cells with OTA (1 µM) had no effect on ERK2 phosphorylation in CHO-OTR cells, but pre-treatment with OTA for 15 min completely blocked the stimulation by 50 nM OT (Fig. 3B).

Roles of Ga and PLC in OT-induced ERK2 phosphorylation. Pretreatment of CHO-OTR cells for 18 h with PTX (200 ng/ml) completely inhibited PTX-stimulated
that the OTR-Gi coupled pathway accounts for part of
after addition of OT (Fig. 4).

These results indicate
A coupled to Gq/11. U-73122 (2.5 µM) inhibited ERK2
the activity of PI-PLC (27), an effector enzyme that is

ADP ribosylation of CHO-OTR cell membranes, without
any apparent effect on cell viability (data not shown). This same dose of PTX inhibited OT-induced
phosphorylation of ERK2 by 25.6 ± 8% (n = 3) by 3 min
after addition of OT (Fig. 4A). These results indicate
that the OTR-Gi coupled pathway accounts for part of
the amount of ERK2 phosphorylated. To determine
whether OT-induced ERK2 phosphorylation is primarily
mediated by G_{q11}, we utilized U-73122, which blocks
the activity of PI-PLC (27), an effector enzyme that is
coupled to G_{q11}. U-73122 (2.5 µM) inhibited ERK2
phosphorylation by 75.1 ± 2.2% (n = 3) by 3 min after
OT stimulation (Fig. 4B). Because the level of inhibition
by U-73122 was greater than that seen with PTX, it is likely that G_{q11} primarily mediates OT stimulation of
ERK2 phosphorylation.

Effects of Ca^{2+} and PKC on OT-elicited ERK2
phosphorylation. Activation of PI-PLC results in an increase
in inositol trisphosphate concentration, resulting in
increased release of Ca^{2+} from intracellular stores, and
in increased DAG concentrations. Rises in both intracel-

ular Ca^{2+} and DAG lead to activation of PKC. Elevation of [Ca^{2+}] by treatment of CHO-OTR cells with the
Ca^{2+} ionophore A-23187 (Calcimycin) for 5 min resulted
in the same level of ERK2 phosphorylation as seen
after OT treatment (Fig. 5A). These findings suggest
that elevation of [Ca^{2+}], after OT stimulation might
result in ERK2 phosphorylation. Indeed, incubation of
cells with intracellular Ca^{2+} sponges, such as

BAPTA-AM (10 µM) and TMB-8-HCl (10 µM) inhibited
OT-induced ERK2 phosphorylation by 51 and 57%,
respectively (Fig. 5B). It is possible that the effects of intracellular Ca^{2+} are
mediated by PKC or are the result of activation of the
calmodulin (CaM)-dependent MLC kinase (23). These
results suggest that the effects of modulators of [Ca^{2+}],
occur through PKC. As would be expected from these

Effect of Ca^{2+} on OT-stimulated ERK2
phosphorylation. CHO-OTR cells were treated either
for 3 min with 50 nM OT, for 5 min with 10 µM Ca^{2+}
ionophore A-23187 (Calcimycin), or for 5 min with Calcimycin after pretreat-
ment for 30 min with the PKC inhibitor GF-109203X (5 µM).
Unphosphorylated ERK1 (pp44MAPK) was also detected. B: effects of
pretreatment of CHO-OTR cells with Ca^{2+} sponges on OT-stimulated
ERK2 phosphorylation. Cells were pretreated with either 1,2-bis(2-
aminoethoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) (10 µM)
or 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-
8)-HCl (10 µM) for 30 min followed by either no (−) or 50 nM OT
treatment for 3 min (+). C: inhibition of OT-stimulated ERK2
phosphorylation by increasing doses of GF-109203X. Cells were
either untreated (control) or pretreated with GF-109203X for 30 min,
followed by stimulation with 50 nM OT for 3 min.
results, OT stimulation of ERK2 phosphorylation was completely blocked by 1 µM GF-109203X (Fig. 5C). Half-maximal inhibition of phosphorylation was obtained with about 200 nM GF-109203X, about ten times greater concentration than the IC50 for PKC inhibition in human platelets and Swiss 3T3 fibroblasts (30). The IC50 for GF-109203X inhibition of PKA, however, is about 2 µM (30).

Effect of OT on [3H]thymidine incorporation into CHO-OTR cells. Although OT has not been considered to be a growth-promoting agent, activation of the ERK is generally associated with cell proliferation. To determine if OT stimulates mitogenesis, [3H]thymidine incorporation was measured after addition of OT. This work was carried out under conditions where OT stimulates ERK2 phosphorylation (in virtually confluent cells). Known mitogens such as 5% FBS and bFGF caused 5.7 ± 0.3- and 1.8 ± 0.2-fold (n = 3) increases in the incorporation of [3H]thymidine by 4 h, respectively (Fig. 6). In contrast, the addition of OT under the same conditions had no effect on [3H]thymidine incorporation (Fig. 6).

MAPK kinase (MEK1/2) inhibition completely blocks OT-induced ERK2 phosphorylation, transcription of c-fos mRNA, and PGE2 synthesis. In view of the lack of effect on OT on CHO-OTR cell proliferation, we examined other possible sites of action. Ternary complex factors, one of which is Elk-1, have been implicated in mediation of c-fos induction (19). MAP kinase catalyzed phosphorylation of Elk-1 allows the Elk-1-serum response factor complex to bind to serum response elements and thereby to activate c-fos expression. OT treatment of CHO-OTR cells induced synthesis of c-fos mRNA within 30 min of treatment (Fig. 7B). To determine the importance of OT-activated ERK2 phosphorylation on c-fos mRNA levels, we used PD-98059, a highly selective inhibitor of MAP kinase kinase (MEK1/2) activation and the MAPK cascade. This agent binds to the inactive forms of MEK and prevents its phosphorylation by either c-Raf or MEKK1 with IC50 values of 5–10 µM (25). Complete inhibition of OT-induced ERK2 phosphorylation was obtained with 10 µM PD-98059 (Fig. 7A). Treatment of CHO-OTR cells with 20 µM PD-98059 resulted in 90 and 97.2% reductions in c-fos mRNA levels (normalized to β-actin mRNA levels) at 30 and 60 min after addition of OT, respectively (Fig. 7B).

CHO-OTR cells responded to OT by rapidly producing PGE2. There was up to a 10-fold increase in PGE2 release by 3 min after addition of 50 nM OT (Fig. 8A). The effect of OT was completely blocked by pretreatment with 1 µM PD-98059 (Fig. 8A). OT stimulation of PGE2 synthesis is therefore mediated by ERK2 phosphorylation. This conclusion is supported by the findings that PTX, which partially inhibited ERK2 by blocking activation of Gαi by OT, also partially inhibited OT-stimulated PGE2 synthesis (Fig. 8B). The effects of PTX on PGE2 synthesis were more apparent at earlier time points (Fig. 8B), suggesting that with time compensatory activation of PTX-insensitive Gα11 can override PTX inhibition. Inhibition of PKC activity with 1 and 5 µM GF-109203X, which results in a total block of OT-stimulated ERK2 phosphorylation (Fig. 5A), resulted in total inhibition of OT-stimulated PGE2 synthesis (Fig. 8C). These experiments show that inhibition of OT-stimulated ERK2 phosphorylation causes concomitant inhibition of OT-stimulated PGE2 synthesis.

**Fig. 6.** Lack of effect of OT on DNA synthesis in CHO-OTR cells. Cells were incubated for 16 h with α-MEM and antibiotics alone (basal) or basal medium containing either 50 nM OT, 5% fetal bovine serum (FBS), or 100 ng/ml basic fibroblast growth factor (bFGF). [3H]Thymidine was then added and allowed to incorporate for 4 h. Results, expressed as counts per minute per well, are shown as means ± SE of triplicate determinations. Basal (0) and OT treatment values were not different when assessed by two-way ANOVA. Both FBS and bFGF values were different from basal values in two-way ANOVA, followed by t-test analysis (⁎ P < 0.05). Similar results were obtained from 2 other independent experiments.

**Fig. 7.** Effects of MEK1/2 inhibition by PD-98059 on OT-stimulated ERK2 phosphorylation and induction of c-fos mRNA expression. A: CHO-OTR cells were pretreated for 1 h with increasing concentrations of PD-98059 and then stimulated for 3 min with 50 nM OT. OT-stimulated ERK2 phosphorylation was assessed by immunoblotting. B: Northern blot analysis of c-fos mRNA after OT treatment for 0, 30, and 60 min, with and without pretreatment for 1 h with 20 µM PD-98059. Percent reduction in c-fos mRNA levels was determined densitometrically after normalization to β-actin mRNA levels.
Our previous studies and those of others have shown that the OTR is functionally coupled to both Gi and Gq in rat myometrium (28). Our present findings show that in CHO cells transfected with the rat OTR, both Gi and Gq interact with the receptor. The two are coupled in about the same proportion as seen in rat myometrium. Other studies have shown that OTR in PG responsive cells such as endometrium and amnion are coupled to G proteins involved in PLC and PKC activation (17, 22). The CHO-OTR cell system thus represents a good model for studying OT action, both from the standpoint of G protein isoforms coupled to the OTR and the cellular response elicited by OT. Stimulation of these cells with OT resulted in the specific phosphorylation and activation of ERK2. In a given cell, several signal transduction pathways involving different heterotrimeric G proteins operate in parallel, and there is increasing evidence that the G protein-mediated signal transduction system undergoes adaptation and cross-regulation at many different levels. Our findings show that both Gi and Gq mediate the effects of OT on ERK2 phosphorylation. Whereas OT-stimulated p42 MAPK activity in human myometrial cells was completely abolished by pretreatment of the cells with PTX (100 ng/ml) for 4 h (24), we found that in CHO-OTR cells the Gq-specific pathway (PTX sensitive) accounted for only about one-quarter of phosphorylated ERK2. Gq activation likely is responsible for the rest, as the PLC inhibitor U-73122 blocked ERK2 phosphorylation by about 75% at a concentration of 2.5 µM. This dose is near that reported for the IC50, at least in some systems (35), suggesting that complete inhibition might have been obtained with greater doses. Unfortunately, because of the limited solubility of the drug, we could not obtain greater concentrations without getting a nonspecific vehicle-related inhibition of ERK2 phosphorylation. Although we have not carried out detailed studies, it appears that the Gi- and Gq-mediated pathways converge at PLC, because inhibition of PKC activity (resulting from PLC activation) with GF-109203X completely blocked OT-stimulated ERK2 phosphorylation. Thus the potential for βγ-subunits of Gi to activate a p21ras-dependent pathway, independent of PKC, appears to be less important in CHO-OTR cells after OT treatment. The findings are in accord with the demonstration by several laboratories that Gβγ-subunits activate PLC directly (16). GF-109203X is highly spe-
cific for the α, β, and γ isoforms of PKC, but it has recently been shown that it also inhibits MAPKAP kinase-1β (Rsk-2) and p70 S6 kinase, both members of the MAPK cascade (2). Because both these activities are downstream from ERK2, it is more likely that the inhibition of OT-stimulated ERK2 phosphorylation by GF-109203X was due to the inhibition of PKC activity.

Inhibition of increases in [Ca^{2+}]_i with the Ca^{2+} sponges partially blocked OT-stimulated ERK2 phosphorylation. Conversely, elevation of [Ca^{2+}]_i with Calcimycin caused ERK2 phosphorylation, indicating that intracellular Ca^{2+} is important in activating ERK2 in CHO-OTR cells. The effects of Calcimycin on ERK2 were blocked by PKC inhibition, suggesting that rather than [Ca^{2+}], causing ERK2 phosphorylation via a PKC-independent, p21ras-mediated process (14, 15), Ca^{2+} activates PKC.

The transcription factor Elk-1 (ternary complex factor-1), which participates in formation of a ternary complex with the serum response element allowing transcription of c-fos, is phosphorylated by ERKs (19). Because OT activated ERK2, it was not surprising that OT also induced increases in c-fos mRNA levels. Indeed, OT induction of c-fos mRNA was virtually eliminated by pretreating CHO-OTR cells with the MEK1/2 inhibitor PD-98059. AP1 activity often is induced by mitogens, and the ERK pathway is generally considered to be involved in cell proliferation or differentiation, depending on cellular context (12). However, OT had no effect on [3H]thymidine incorporation by PKC. Thus the role of OT-induced synthesis of c-fos mRNA in CHO-OTR cells remains to be determined. The phosphorylation of ERK2 is critical for OT-induced PGE_2 synthesis, as the effects of OT on PGE_2 were completely blocked by PD-98059. Inhibition of ERK2 phosphorylation by PTX and GF-109203X gave proportional effects on OT-stimulated PGE_2 release, indicating a clear causal relationship between ERK2 activation and PGE_2 synthesis. Although it has been demonstrated that MAPK is involved in cPLA_2 phosphorylation (23), there is evidence to suggest that the rise in intracellular Ca^{2+}, and not phosphorylation of cPLA_2, is essential for activation of the arachidonic acid cascade in rat liver macrophages (5). In other studies, PD-98059 inhibited p42/p44MAPK activation in thrombin-, collagen- and phorbol ester-stimulated platelets, but did not interfere with the release of arachidonic acid or with cPLA_2 phosphorylation (7). Our findings, which indicate a causal relationship between inhibition of ERK2 phosphorylation and inhibition of PGE_2 production, are more consistent with the conclusions of other studies (34). In summary, we have shown that PKC activation through G proteins and ERK2 phosphorylation are vital steps in the process of OT stimulation of PGE_2 and c-fos expression (Fig. 9). PKC-independent steps involving p21ras appear to be less important in OT signaling in CHO-OTR cells (Fig. 9). Our studies also establish these cells as a valid model to study additional signaling pathways involved in OT action.

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