Role of MAP kinase phosphatases in GnRH-dependent activation of MAP kinases

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Abstract

GnRH controls the synthesis and release of the pituitary gonadotropic hormones. MAP kinase (MAPK) cascades, including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways, are crucial for GnRH-induced gene activation. In the present study, we investigated the function of GnRH-induced MAPK phosphatases (MKPs) using an in vivo mouse model as well as the αT3–1 cell line. Following GnRH agonist stimulation, in vivo gene profiling demonstrated that both MKP-1 and MKP-2 are induced with distinct temporal profiles, suggesting differential roles of these MKPs in the regulation of MAPK activation. Elevated activity of MKP-2 in αT3–1 cells, through either overexpression or activation of the endogenous MKP-2 gene, was correlated with inhibition of GnRH-induced activation of ERK and JNK, as well as the expression of ERK- and JNK-dependent proto-oncogenes. These data supported the conclusion that GnRH-induced MKPs likely serve as negative feedback regulators that modulate MAPK activity and function in the GnRH signaling pathway.

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Introduction

Gonadotropin-releasing hormone (GnRH) activates the synthesis and release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Gharib et al. 1990). LH and FSH play critical roles in the control of testicular and ovarian gametogenesis and steroidogenesis. GnRH is released from the hypothalamus in a pulsatile manner to regulate pituitary gonadotropin function (Clarke 1987, Clarke & Cummins 1987). The intracellular effects of GnRH are mediated by a serpentine receptor coupled to a heterotrimeric G protein in the Gα11 family (Hsieh & Martin 1992, Stanislaus et al. 1998). Activated Gαq stimulates phospholipase Cβ, which leads to the generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces release of Ca2+ from intracellular stores, resulting in cytosolic Ca2+ oscillations. Ca2+ mobilization in gonadotropes plays a role in the secretion of gonadotropic hormones and transcriptional regulation of glycoprotein hormone subunit genes. DAG activates protein kinase C (PKC) isozymes, which are linked to transcriptional activation of the α subunit gene and proto-oncogenes regulated primarily by members of the MAP kinase (MAPK) family (Česnjaj et al. 1993, Mitchell et al. 1994, Roberson et al. 1995, Sundaresan et al. 1996, Reiss et al. 1997) (for reviews see Stojilkovic et al. 1994, Hille et al. 1995, Naor et al. 1998).

Three major classes of MAPKs, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, are activated following GnRH receptor occupancy in pituitary gonadotropes (Roberson et al. 1995, Sundaresan et al. 1996, Reiss et al. 1997). Activation of MAPK requires dual phosphorylation of threonine and tyrosine residues in the conserved TXY motif by MAPK kinase (Cobb & Goldsmith 1995). A family of dual specificity protein phosphatases (MAPK phosphatases or MKPs) selectively dephosphorylate MAPKs in vitro and in vivo by dephosphorylation of both tyrosine and threonine residues (hence the designation of dual specificity; Farooq & Zhou 2004). MKPs have overlapping yet unique patterns of tissue distribution, subcellular localization (nuclear versus cytosolic), substrate specificity and response to mitogenic stimuli, suggesting that they may have distinct physiological functions (Lewis et al. 1998). The founding member of the family, MKP-1, is widely expressed in many proliferating and terminally differentiated cell types (Janknecht & Nordheim 1992, Janknecht et al. 1992, 1994). In most cell lines studied to date, MKP-1 is activated as an immediate early gene in response to various stimuli, such as growth factors (Lau & Nathans 1985), insulin (Kusari et al. 1997) and u.v. light (Liu et al. 1995, Bokemeyer et al. 1996). Several studies suggest that MKP-1 induction is ERK- or JNK-dependent and functions as a negative feedback mechanism to inactivate MAPKs (Bokemeyer et al. 1996, Brondello et al. 1997). A closely related MKP family member, MKP-2, is also widely expressed in a variety of cell lines and tissues. In pituitary...
gonadotropes, GnRH up-regulates the expression of MKP-2 through activation of the immediate early gene early growth response factor 1 (Egr-1) (Roberson et al. 1995, Zhang et al. 2001b). Induction of MKP-2 expression by GnRH is dependent on MAPK activation (Zhang et al. 2001a), suggesting potential feedback regulation within the GnRH signaling pathway by MKP-2. In the present study, we examined the role of MKP-1 and MKP-2 in the regulation of MAPK activity by GnRH. Using both an in vivo mouse model and the αT3–1 cell line, we observed distinct temporal profiles for the induction of MKP-1 and MKP-2 following GnRH treatment. Expression of MKP-1 and MKP-2 correlated with the activation and decline of MAPK activity, suggesting a role for these MKPs in the regulation of magnitude as well as the duration of MAPK activation. Through manipulation of MKP expression levels in αT3–1 cells, we have shown that elevated MKP-2 was highly correlated with down-regulation of ERK and JNK activity as well as ERK- and JNK-dependent gene expression following GnRH stimulation. Overall, these studies support a role for MKPs as negative feedback regulators in the GnRH signaling pathway in the pituitary gonadotrope.

Materials and methods

Materials

The c-Fos, c-Jun, ERK2, JNK1, MKP-1 and MKP-2 antibodies, horseradish peroxidase (HRP)-conjugated secondary antibody and protein A/G-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific (dual phosphorylated) ERK antibody was purchased from Promega (Madison, WI, USA). Phospho-specific JNK and MEK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA). The MEK1 antibody was from Transduction Laboratories (Lexington, KY, USA). Phorbol 12-myristate 13-acetate (PMA) and the GnRH agonist (GnRHa) buserelin were purchased from Sigma. Phospho-specific JNK and MEK1/2 antibodies, horseradish peroxidase (HRP)-conjugated secondary antibody and protein A/G-agarose were purchased from Promega (Madison, WI, USA). Phospho-specific (dual phosphorylated) ERK antibody was purchased from Promega (Madison, WI, USA). Phospho-specific JNK and MEK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA). The MEK1 antibody was from Transduction Laboratories (Lexington, KY, USA). Phorbol 12-myristate 13-acetate (PMA) and the GnRH agonist (GnRHa) buserelin were purchased from Sigma. Bacterial expression vector for Glutathione S-transferase-activating transcription factor 2 (GST-ATF2) was from Dr Michael Green (University of Massachusetts, Worcester, MA, USA).

Cell culture

αT3–1 cells were a generous gift from Pamela Mellon (UC San Diego, CA, USA) and were grown to approximately 50% confluence in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cells were then serum starved for 2 h in DMEM prior to administration of hormones and various agents.

In vivo mouse model for GnRH action and transcript profiling of MKPs

All experimental protocols used in these studies were approved by the Cornell University Institutional Animal Care and Use Committee. Female CF-1 mice (Charles River, Wilmington, MA, USA) were ovariectomized at 30 days of age and allowed 7–10 days recovery time. All animals were passively immunized against endogenous GnRH as has been described previously (Duval et al. 2000). Seventy-two hours later, mice were administered saline as control or 100 ng dAla⁶-GnRH (Sigma), an agonist of GnRH not recognized by the immunoneutralizing antibody. All mice were killed by CO₂ asphyxiation. Control animals (n=6) were killed 120 min after saline injection. Mice receiving the dAla⁶-GnRH agonist were killed 20, 40, 60 and 120 min following injection (n=6/time-point). Levels of LH in trunk blood were measured by specific RIA (Duval et al. 2000). Individual time-points were compared with the zero time-point by paired t-test and Bonferroni’s procedure to determine statistical difference in serum concentrations of LH. Total cellular RNA was isolated from whole pituitaries using the Absolutely RNA isolation kit (Stratagene, La Jolla, CA, USA) as described by the manufacturer including treatment with DNase. DNA-free RNA samples isolated from individual animals were used in subsequent gene profiling studies described below.

SYBR green-based real-time PCR (qPCR) was performed as previously reported (Wurmbach et al. 2001). In brief, total RNA was converted into cDNA and ~250 pg was used for 40-cycle three-step PCR in an ABI Prism 7900HT (PE Applied Biosystems, Foster City, CA, USA) in 20 mM Tris, pH 8.4, 50 mM KCl, 3 mM MgCl₂, 200 μM deoxynucleoside triphosphates, 0.5 SYBR Green I (Molecular Probes, Inc., Eugene, OR, USA), 200 nM of each primer and 0.5 U Platinum Taq (Invitrogen). Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. The relative levels of expression were interpolated from detection threshold (CT) values normalized relative to the CT of ribosomal protein S11 (RPS11), an unregulated housekeeping gene. Egr-1 and MKP transcripts in each sample were assayed twice, and the average CT values were used to calculate the fold change ratios between experimental and control samples for each gene used in the analysis. For statistical analysis, fold change values were log transformed and subjected to a paired t-test (comparing each time-point to time zero) followed by Bonferroni’s procedure.

Western blot analysis

αT3–1 cells were cultured in 60 mm plates and treated as described. At the end of each experiment, cells were
washed once with cold HEPES-buffered saline (20 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄ and 0·1% dextrose) and lysed in RIPA buffer (20 mM Tris–HCl, pH 8·0, 137 mM NaCl, 10% glycerol, 1% NP-40, 0·1% SDS, 0·5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0·2 mM phenylmethylsulfonyl fluoride and 5 mM benzamidine). Following centrifugation to remove cell debris, the whole cell lysates were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% non-fat dry milk (NFDM) in TBST (10 mM Tris–HCl, pH 7·6, 150 mM NaCl and 0·05% Tween-20) and then probed with primary antibody. MKP-1 and MKP-2 antibodies were diluted 1:100 in 5% NFDM/TBST, c-Fos antibody was diluted 1:500 in 5% NFDM/TBST, c-Jun, MEK1 and ERK2 antibodies were diluted 1:1000 in 5% NFDM/TBST, phospho-specific ERK and JNK antibodies were diluted 1:20 000 and 1:1000 (respectively) in 0·1% bovine serum albumin (BSA)/TBST, and phospho-MEK1/2 antibody was diluted 1:1000 in 5% BSA/TBST. The above antibodies were incubated with the blots for either 2 h at room temperature or overnight at 4 °C. At the end of the incubation, the blots were washed in TBST, and then incubated with HRP-conjugated secondary antibody (1:5000 dilution in 5% NFDM/TBST) for 30–60 min. After four washes in TBST, signals were detected using enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA, USA).

JNK kinase assay

JNK kinase assay was performed as previously described (Mulvaney et al. 1999, Mulvaney & Roberson 2000). Briefly, whole cell lysates were prepared in the same way as described for western blot analysis. JNK activity was immunoprecipitated with JNK1 antibody and protein A/G-agarose. The kinase reaction was carried out at 30 °C for 30 min using GST-ATF2 as substrate. After resolving by 10% SDS-PAGE, the phosphorylated GST-ATF2 was visualized by autoradiography. In some experiments, phospho-specific JNK antibody and Western analysis was substituted for the in vivo kinase assay for ease of experimentation. Results from the JNK catalytic assay and the phospho-specific antibody were similar. In some cases for both the ERK and JNK data, individual bands at time zero and 30 min following GnRH administration were quantitated using densitometry and data were compared by analysis of variance and Tukey’s Studentized range test.

Transient transfection

αT3–1 cells were cultured in 60 mm plates to 20–50% confluence before transfection. The expression vector for MKP-2 was co-transfected into αT3–1 cells with the expression vectors for ERK and JNK using SuperFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. The total amount of DNA was kept constant by adding appropriate amounts of pcDNA3 vector. Total DNA (12 µg) and SuperFect reagent (50 µl) (Qiagen) were used in each transfection. Whole cell lysates were collected 36 h after the transfection and western blot analysis was performed as described above.

Results

Temporal correlation between the induction of MKP-1, MKP-2 and activation of MAPKs

Induction of MKPs by GnRH suggests a negative feedback mechanism that controls MAPK activity in pituitary gonadotropes. To investigate this possibility, we examined the activation profile of these signaling components following GnRH treatment. First, we studied MKP-1, MKP-2 and Egr-1 mRNA expression using an in vivo mouse model for GnRH pulse stimulation. Passive immuno-neutralization of endogenous GnRH in ovariectomized female CF-1 mice was passively immunized against endogenous GnRH. Seventy-two hours later, the mice were administered 100 ng dAla⁶-GnRH. Trunk blood samples were collected at the indicated time-points and levels of LH were measured by RIA. Data are presented as means±s.e.m. (n=6/time-point). **P<0·05 compared with time zero.

Figure 1 LH secretion following dAla⁶-GnRH agonist treatment in mice. Ovariectomized female CF-1 mice were passively immunized against endogenous GnRH. Seventy-two hours later, the mice were administered 100 ng dAla⁶-GnRH. Trunk blood samples were collected at the indicated time-points and levels of LH were measured by RIA. Data are presented as means±s.e.m. (n=6/time-point). **P<0·05 compared with time zero.
administration, LH secretion increased markedly and remained elevated for the duration of the study (Fig. 1). Correlated with the LH secretory response, mRNA levels for Egr-1, MKP-1 and MKP-2 were increased albeit with differing kinetics (Fig. 2). MKPs and Egr-1 mRNA have been shown to be up-regulated by GnRH using the LβT2 cell model and array/qPCR approaches (Wurmbach et al. 2001). Egr-1 was shown to be necessary for the expression of the LHβ subunit promoter and Egr-1 null mice are infertile due to lack of LH (Lee et al. 1996, Topilko et al. 1998). Our laboratory has shown previously that Egr-1 is also involved in the regulation of the MKP-2 gene promoter in αT3-1 cells; (Zhang et al. 2001b). In vivo, Egr-1 mRNA levels increased 11·7 ± 1·5-fold (means ± s.e.m; P<0·05) by 20 min following the dAla⁶-GnRH pulse, and declined to baseline by 120 min. The kinetics of MKP-1 mRNA expression was similar to Egr-1 with a rapid induction (5·8 ± 0·5-fold; P<0·05) followed by a return to baseline by 40 min after the dAla⁶-GnRH pulse. In contrast, MKP-2 mRNA was elevated 1·98 ± 0·53-fold (P<0·07) at 60 min following the dAla⁶-GnRH pulse. Thus, two overlapping mRNA profiles exist for MKP expression induced by GnRH in vivo, a rapid rise in MKP-1 expression followed by a later increase in the expression of MKP-2 mRNA. Importantly, these mRNA induction profiles were consistent with the protein expression profiles observed in αT3-1 cells previously (Zhang et al. 2001a).

Next, we examined the time-course of MKP-1 and MKP-2 protein expression and ERK and JNK phosphorylation/activation in αT3-1 cells following treatment with the GnRH agonist buserelin (Fig. 3). Consistent with the in vivo mRNA data, the expression of MKP-1 and MKP-2 proteins temporally overlapped with each other, but each followed a distinct time-course. After GnRH agonist treatment, MKP-1 protein amounts increased to high levels within 30 min, peaked at 1 h, and had declined to near baseline within 4 h. MKP-2 protein amounts increased gradually and reached the peak level 2 h after GnRH treatment. MKP-2 protein declined gradually thereafter, but remained at measurable levels 8 h following hormone treatment (data not shown). Comparing in vivo mRNA data (Fig. 2) with protein data (Fig. 3) reveals that much greater differences exist when examining expression of MKPs at the protein levels. This may reflect differences comparing in vivo conditions with clonal αT3-1 cells. We also cannot rule out the possibility that there may be differential regulation of MKPs at the transcriptional and translational levels. When the activation time-course of the MKPs was compared with that of ERK and JNK following GnRH agonist administration, the maximal level of MKP-2 appears to be temporally associated with the apparent dephosphorylation/inactivation of ERK and JNK (Fig. 3). The phospho-specific ERK antibody requires
dual phosphorylation of ERK for immunoreactivity, thus reflecting the activation state of the ERK enzymes. These correlative data were consistent with a role of MKP-2 in controlling the duration of GnRH-induced MAPK activation. MKP-1 expression overlapped with the peak activity of both ERK and JNK, suggesting that MKP-1 may regulate the magnitude as well as the duration of MAPK activation upon GnRH stimulation.

Effects of MKP-2 overexpression on the activation of ERK and JNK in αT3–1 cells

Our results in Fig. 3 demonstrate a strong temporal correlation between the induction of MKP-2 and the inactivation of ERK and JNK. Based on this observation, we further tested the function of MKP-2 in GnRH signaling pathways using overexpression studies in αT3–1 cells. Exogenous overexpression of MKP-2 primarily inhibited GnRH-induced phosphorylation/activation of JNK and to a lesser extent ERKs (Fig. 4). At a higher dose of MKP-2 expression vector, induction of JNK catalytic activity was reduced (control 2.58 ± 0.3-fold versus MKP-2 overexpression 1.34 ± 0.1-fold at the 30-min time-point; \( P < 0.05 \)), while ERK dephosphorylation was numerically reduced (control 5.11 ± 0.4-fold versus MKP-2 overexpression 2.96 ± 0.6-fold); however, this difference did not reach statistical significance. These results provide direct evidence that elevated expression of MKP-2 inhibits GnRH signaling through JNK in αT3–1 cells. Together with the MKP-2 expression profile following GnRH treatment (Figs 2 and 3), these data are consistent with a role of MKP-2 as a negative feedback regulator of JNK-dependent GnRH signaling.

Down-regulation of MAPK activity by GnRH-induced MKP-2 in αT3–1 cells

GnRHa pulse pretreatment elevates endogenous MKP-2 levels

To further investigate the function of GnRH-induced MKP-2, we sought to examine GnRH-dependent MAPK activation in the presence of elevated endogenous MKP-2 levels. As shown in Fig. 3, 4 h after GnRHa stimulation MKP-2 protein remained at maximal levels while ERK and JNK activity declined to near basal levels. This allowed us to compare ERK and JNK activation by GnRHa during times when cells have low amounts of MKP-2 (time 0) and times when MKP-2 amounts were markedly elevated (4 h). Initially, we
examined whether a single 15-min pulse of GnRHa was sufficient to activate MKP-2 expression similar to the effect of chronic GnRHa treatment. The GnRHa pulse treatment had the same effects on the activation of MKP-2 (Fig. 5A), ERK and JNK (data not shown) as the chronic hormone treatment. The 15-min GnRHa pulse provided an experimental paradigm that resulted in minimal hormone-induced down-regulation of GnRH receptors (Hawes & Conn 1992) which was an important consideration for subsequent studies.

We then compared the expression of MKP-1 and MKP-2 following the GnRHa pulse treatment. Consistent with the data shown in Fig. 3, 4 h after the GnRHa pulse MKP-1 levels were basal while MKP-2 levels were near maximum (Fig. 5B). These control studies support the notion that MKP-1 would have minimal contribution to MAPK inactivation within this time-frame. Conversely, MKP-2 induced by GnRH would likely play a prominent role in MAPK inactivation 4 h following a GnRHa pulse pretreatment.

Figure 5 Chronic and 15-min pulse treatment of GnRHa had similar effects on MKP induction. (A) MKP-2 induction was examined following chronic or pulsatile GnRHa treatment. In parallel studies, groups of αT3-1 cells were treated with GnRHa (10 nM) either chronically or as a 15-min pulse (beginning at time 0) followed by washing and maintenance in hormone-free medium. Cells were collected at the time-points indicated. MKP-2 protein amounts were examined by western blot analysis. (B) αT3-1 cells were treated with a 15-min GnRHa pulse as described in (A). Whole cell lysate was collected at 0 and 4 h after the treatment, and MKP-1 and MKP-2 protein amounts were analyzed by Western blotting. These studies were completed at least three times on separate occasions.

Figure 6 Elevated expression of endogenous MKP-2 was correlated with reduced MAPK activation by GnRHa. αT3-1 cells were treated with or without a 15-min pulse of GnRHa (10 nM) as described in Fig. 5. Four hours following the GnRHa pulse, the cells were stimulated again with GnRHa (10 nM) for the times indicated. Phospho-ERK and total ERK amounts were determined by western blotting. JNK activity was measured by immuno-precipitation followed by kinase assay. These studies were completed at least three times on separate occasions.

GnRHa pulse pretreatment inhibits ERK and JNK activation as well as ERK- and JNK-regulated gene expression

To examine the effect of the GnRHa pulse pretreatment on MAPK activation, αT3-1 cells were treated with or without a single 15-min pulse of GnRHa, followed by wash out of the hormone. Four hours later, the cells were stimulated again with GnRHa and the activation of ERK and JNK was examined. In pre-pulsed cells, GnRHa-induced ERK and JNK phosphorylation/activation was reduced in magnitude, and JNK activity was shortened in duration by ~1 h (Fig. 6). These studies were consistent with overexpression of MKP-2 relative to JNK activity (Fig. 4). Interestingly, inhibition of ERK phosphorylation was more evident in these studies using the GnRHa pulse paradigm. The possibility exists that endogenous MKP-2 expression levels were greater relative to levels achieved using overexpression of the MKP-2 expression vector in earlier studies. Thus, within the context of elevated levels of endogenous MKP-2 and basal levels of MKP-1, GnRHa-induced activation of ERK and JNK was attenuated.

Next, we examined the effect of the GnRHa pulse pretreatment on the activation of ERK- and JNK-regulated immediate early genes, c-Fos and c-Jun. When the same pulse paradigm as in Fig. 6 was used, GnRHa induction of c-Fos expression 4 h following the initial GnRHa pulse treatment was profoundly reduced in αT3-1 cells (Fig. 7), c-Jun is a specific substrate for the JNK pathway. The phosphorylation state of c-Jun was examined by western blot analysis where phosphorylated c-Jun migrates more slowly on SDS-PAGE. Consistent with the reduction in JNK activation, phosphorylation of c-Jun and up-regulation of c-Jun protein amounts were greatly reduced by the GnRHa pulse pretreatment (Fig. 7). Overall, these studies provided correlative data to support the notion that
endogenous phosphatase activity including MKP-2 may serve as a negative feedback regulator of ERK and JNK functions in the GnRH signaling pathway.

The reduced activation of ERK in the pre-pulsed cells is not due to upstream desensitization events

To verify that the reduced effect of the second GnRHa treatment on ERK activation was not caused by possible desensitization of upstream signaling events, we conducted two control studies. In one study, GnRHa was used for the first pulse treatment to elevate MKP-2 protein amounts; however, phorbol ester was used for the second stimulation effectively bypassing the GnRH receptor and signaling components upstream of PKC. The GnRHa pulse pretreatment had a similar inhibitory effect on PMA-induced ERK activation (Fig. 8A). This result indicated that signaling events downstream of PKC activation were attenuated by the pulse pretreatment.

In the second study, we examined the activation of MEK1/2, the immediate upstream activators of ERK1 and ERK2, in the same pulse paradigm as used in Fig. 6. The pulse pretreatment did not alter the activation pattern of MEK1/2 up to 2 h following the second GnRHa treatment (Fig. 8B). These data strongly argue that the reduced activation of ERKs in the pre-pulsed cells was not due to desensitization events that might occur upstream of ERK activation. Taken together, our data suggest that elevated endogenous dual specificity phosphatases (such as MKP-2) in the GnRHa-pretreated cells may play a role in inhibition of ERK activation by the second GnRHa stimulation.

### Discussion

MAPK cascades are key components of signaling pathways that integrate extracellular stimuli to nuclear events such as gene transcription (for reviews see Cobb & Goldsmith 1995, Lewis et al. 1998). MAPK activity is determined by the balance between specific activating kinases and inactivating phosphatases. Dual-specificity MKPs have been shown to selectively inactivate MAPKs. MKP activities are controlled primarily at the level of gene expression (for review see Lewis et al. 1998). In vivo induction of MKP expression by dAla6-GnRH in the mouse model (Figs 1 and 2) confirms our previous report on the regulation of MKPs in both cultured primary gonadotropes and αT3–1 cells. These in vivo results suggest that both MKP-1 and MKP-2 may be crucial effectors in the GnRH signaling pathway under physiological conditions.

To further investigate the function of MKPs in GnRH signaling, we used the αT3–1 cell model. The close temporal correlation between GnRHa-induced MKP-1 and MKP-2 expression and inactivation of MAPKs was consistent with the notion that these MKPs regulate the magnitude and duration of MAPK activation. Further, MKP-2 overexpression provided clear evidence that MKP-2 is sufficient to dephosphorylate and thus inactivate the catalytic activity of JNK. Based upon these
observations, we have used a model experimental paradigm in which a GnRHa pulse pretreatment was sufficient to elevate endogenous MKP-2 levels and to attenuate MAPK signaling and proto-oncogene expression following subsequent GnRH stimulation. The expression profile of MKP-2 in this experimental paradigm suggests that it may be an important factor responsible for the reduced activation of MAPKs. These studies provide a novel view of GnRH action in which GnRH desensitizes gonadotrope cell function through activation of MKPs. The mechanisms of cellular desensitization by chronic or acute hormone exposure via serpentine receptors have not been fully described. Chronic exposure to GnRH agonists has been linked to desensitization of GnRH-induced hormone secretion (Chang et al. 1988, Gorospe & Conn 1988, Hawes & Conn 1992, Mason et al. 1994, McArdle et al. 1996) and losses in GnRH receptor mRNA in pituitary gonadotropes (Chang et al. 1988, Pinski et al. 1996). In contrast, secretory and signaling desensitization of gonadotropes has also been observed immediately following acute exposures to GnRH (Anderson et al. 1995, Weiss et al. 1995). Our studies differed from these reports in that a single GnRHa pulse was sufficient to modulate GnRH responsiveness 4 h later. This result suggests that GnRH-inter-pulse interval may be an important determinant of MAPK functions in GnRH-responsive gonadotropes. Additional in vivo studies are required to test this hypothesis.

In conducting the GnRHa pulse studies, one major concern was that response to GnRHa stimulation 4 h following the initial 15-min pulse may be influenced by down-regulation of GnRH receptors after the initial pulse. While we cannot completely discount this possibility, our two control studies support the notion that attenuation of ERK activities following the second agonist stimulation was likely associated with increased cellular amounts of MKP-2 and related dual-specificity phosphatases. When PMA was used as the second agonist in the GnRH pulse paradigm, it functionally bypassed potential desensitization events upstream of the PKC isozymes, including loss of GnRH receptors. In addition, it is unlikely that the GnRHa pulse treatment can cause PKC depletion over the 4-h experimental period since chronic GnRH treatment did not result in significant loss of PKC in αT3–1 cells (McArdle et al. 1987, Kratzmeier et al. 1996). Therefore, the result from the PMA study (Fig. 8A) indicated that signaling components downstream of PKC contribute to the desensitization of ERK activation in response to the initial GnRHa pulse. Consistent with this, our study on MEK1/2 activation (Fig. 8B) also indicated that this desensitization occurs downstream of MEK1/2. Thus, we were able to discount other potential effectors of cellular desensitization by GnRH action, lending support to the hypothesis that accumulation of MKP-2 (or related phosphatases) may play a functional role in determining cellular sensitivity to GnRH action on MAPK-dependent processes.

Our studies with the GnRHa pulse paradigm were consistent with the studies of others in examining the role of MKPs in the down-regulation of MAPK signals. In PC12 cells, induction of MKP-1 and MKP-2 by nerve growth factor blocked u.v. light-induced JNK activation 2 h later (Hirsch & Stork 1997). In a separate study, basic fibroblast growth factor (bFGF) caused prolonged expression of MKP-3, which inhibited ERK activation up to 2 days after the bFGF treatment (Camps et al. 1998). On the other hand, inhibition of MKP-1 expression in rat mesangial cells has been shown to prolong JNK and c-Jun activation and potentiate JNK-mediated apoptosis (Guo et al. 1998). These data, together with our study in αT3–1 cells, provide evidence that MKPs are physiological regulators of MAPK pathways and downstream effectors of these cascades. This idea may be of particular importance to the GnRH system since the inter-pulse interval leading to LH pulsatility can vary greatly depending upon the physiological state.

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