Protein kinase A serves as a primary pathway in activation of Nur77 expression by gonadotropin-releasing hormone in the LßT2 mouse pituitary gonadotroph tumor cell line

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Abstract. Nur77 belongs to a subfamily of nuclear receptors that includes two other members, Nor-1 and Nurr1. It plays an important role in a number of biological processes, including regulation of signaling functions in the hypothalamo-pituitary-adrenal axis, regulation of thymocyte apoptosis, regulation of steroidogenesis and regulation of tumor cell proliferation and apoptosis. In previous studies, using DNA microarray analysis of the effects of the gonadotropin-releasing hormone (GnRH) on the mouse pituitary gonadotroph cell line LßT2, we identified Nur77 as one of the highly regulated immediate early genes involved in this response, with >40-fold up-regulation after 1 h of treatment of the cells with the GnRH agonist [D-Ala^{6}GnRH (GnRHA)]. GnRH is a hypothalamic decapeptide that stimulates the secretion and expression of gonadotropins (follicle stimulating hormone, FSH and luteinizing hormone releasing hormone, LH) from anterior pituitary through activation of high affinity receptors present on cell membrane of pituitary gonadotropes. In addition to pituitary, the presence of GnRH high affinity receptors has been reported in various cancers and cancer cell lines. In addition, GnRH and its analogs are clinically used in the treatment of prostate cancer. To elucidate the molecular mechanism involved in regulation of Nur77 by GnRH, we first confirmed upregulation of Nur77 in response to GnRH analog (GnRHA) in LßT2 cells. Nur77 mRNA was upregulated within 30 min of GnRHA treatment and returned to nearly basal level after 24 h of treatment. Nur77 protein expression was upregulated after 2 h of treatment and remained steady even after 12 h of treatment. The expression of Nur77 mRNA was induced by GnRHA in a dose-dependent manner. Induction of Nur77 expression was stimulated on treatment of cells with forskolin and 8-Br-cAMP, whereas H-89, a specific inhibitor of PKA pathway significantly inhibited GnRHA-induced Nur77 expression. Treatment of cells with both H-89 and EGTA completely blocked the GnRHA-induced expression of Nur77, indicating that both calcium and cAMP/PKA play an important role in regulation of Nur77 expression by GnRHA. Analysis of the protein kinase C (PKC) signaling pathway using specific inhibitors for PKC, Erk1/2, p38 and JNK demonstrated that these pathways are not involved in GnRHA-induced Nur77 expression. Based on our results, we conclude that activation of protein kinase A is the major mechanism regulating the expression of Nur77 by GnRH which may serve as a down-stream signaling gene to mediate the antitumor effects of GnRH.

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Introduction

Nur77 (also known as NGFI-B or TR3) encodes an orphan nuclear receptor that is expressed in a wide variety of tissues, i.e., the thymus, muscle, liver, thyroid, lung, testis, ovary, ventral prostate, adrenal and pituitary (1,2). It belongs to a subfamily of nuclear receptors that includes two other members, Nor-1 (mitogen-induced nuclear orphan receptor [MINOR], nuclear receptor subfamily 4, group A, member 3 [Nr4a3]) and Nurr1 (transcriptionally inducible nuclear receptor [TINUR], nuclear receptor subfamily 4, group A, member 2 [Nr4a2]). The members of this family are classified as immediate early genes as their expression is rapidly but transiently, induced by serum and growth factors (3,4), as well as various other stimuli involved in cellular differentiation and proliferation (5,6). Expression of Nur77 in endocrine cells is rapidly induced by corticotrophin-releasing hormone (CRH), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and adrenocorticotropic hormone (ACTH) (7-10). Previously, Nur77 activation by several apoptosis inducing agents, such as 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes to induce apoptosis in pancreatic cancer cell line (Panc-28) apoptosis has been demonstrated (11).

The domain structure of Nur77 and other family members is that of a classical nuclear receptor structure, with an N-terminal activation function-1 (AF-1) domain, a highly homologous DNA-binding domain (DBD) and a similar ligand-binding domain (LBD), together with a C-terminal activation function-2 (AF-2) domain (reviewed in ref. 12). As a monomer, Nur77 binds to the Nur77-binding response

element (NBRE), which contains the hexanucleotide sequence AGGTCA, a typical recognition motif of the RAR/RXR family (13). Binding of homodimers of Nur77 to DNA has been shown as well as binding of heterodimers composed of Nur77 and the retenoid X receptor (14). The regulation of Nur77 expression is not understood fully; however, recent studies indicate that Nur77 is regulated both at the level of transcription and post-translationally. In addition to direct binding of Nur77 to NBRE, its binding also has been demonstrated to be regulated by recruitment of co-activators and co-repressors (12,15-17).

Nur77 has been implicated in a number of biological processes including regulation of signaling functions in the hypothalamo-pituitary-adrenal axis (18-20) and regulation of steroidogenesis by modulating the expression of steroidogenic enzymes (21). In addition, Nur77 has been shown to be involved in T-cell receptor (TCR) mediated apoptosis in immature thymocytes and T-cell hybridomas (22,23), induction of apoptosis in lung cancer cells (24) and prostate cancer cells (25), activation-induced cell death (11,22,26-28) and neuronal differentiation of PC12 cells (29). In contrast to its role as a pro-apoptotic molecule, Nur77 has also been described as a survival factor when induced through tumor necrosis factor signaling (30). A recent report demonstrates that Nur77 can interact with Bcl-2, resulting in conversion of Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule in HEKT and HCT-116 cells (31). Apart from its role in endocrine signaling and apoptosis, Nur77 has also been shown to be involved in tumor progression and metastasis by its stabilization of hypoxia inducible factor-α (HIF-1α) (32) and stimulation of proopiomelanocortin (POMC) (7) besides being identified as a key 'signature-gene' involved in metastatic progression of tumors (33) and, previously, Nur77 along with and other family members have been shown to play important roles in cellular transformation and oncogenesis of cervical cancer (34).

High levels of expression of Nur77 have been reported in pituitary (35), but the physiological stimuli that regulate its expression in pituitary remains unknown. In our recent studies, using DNA microarray analysis of mRNA expression of mouse pituitary gonadotroph cells (LßT2) on treatment with gonadotropin-releasing hormone (GnRH) agonist (D-Ala^6) GnRH (GnRHA), we found an ~40-fold increase in expression of Nur77 mRNA within 1 h of treatment (36). This finding was consistent with observations made previously by other investigators (3,4,37,38). Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that is synthesized by hypothalamic neurons and released into the portal circulation in a pulsatile fashion (39). It plays an important role in reproductive physiology by regulating the expression and secretion of FSH and LH. GnRH has also been shown to be involved in regulation of cell proliferation and apoptosis (40,41) and cytoskeletal rearrangements (42). GnRH and its analogs are currently used in the treatment of prostate cancer (reviewed in ref. 43). The present studies were carried out to gain an understanding of the regulation of Nur77 expression by GnRH in LßT2 cells and to decipher the molecular signaling mechanism(s) involved in this regulation. Our results indicate that GnRH transiently activates Nur77 expression and this activation by GnRH is primarily achieved through the activation of cAMP-PKA signaling pathway.

Materials and methods

Reagents. GnRH-A ([D-Ala^6]GnRH), 12-O-tetradecanoylphorbol-13-acetate (PMA), H-89, ionomycin, 8-Br-cAMP, GnRH antagonists (Antide) and Forskolin (FSK) were obtained from Sigma (St. Louis, MO). PKC inhibitors BIM-I, BIM-IV and JNK inhibitor SP600125 were purchased from Calbiochem (La Jolla, CA). MEK inhibitor U0126 and p38 inhibitor SB202190 were purchased from Biosource International (Camarillo, CA). Rabbit monoclonal antibodies against Erk1/2, p38 and JNK were obtained from Cell Signaling Technologies (Beverly, MA).

Cell culture. LßT2 cells were maintained in monolayer cultures in DMEM supplemented with 10% FBS and antibiotics in humidified 5% CO_2 at 37˚C (44). After 24 h of plating, the medium was replaced with serum-free medium and incubated for an additional 24 h. The cells were then treated with GnRHA, various activators, or inhibitors as detailed in figure legends.

Isolation of total RNA and reverse transcriptase (RT) PCR. Total RNA from LßT2 cells was purified using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The RNA pellets were resuspended in RNase-free water and any contaminating DNA removed from the preparations by treatment with DNaseI. The yield of total RNA was measured using spectrophotometery and the quality of preparation checked by 1% agarose. First-strand cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA), as described previously (45). PCR primers (sense 5'-GTGCTCCTCAGCTTGTTCC-3' and antisense 5'-CTGACACGAAGCATTGTCAC-3') for Nur77 were designed, based on the mouse Nur77 coding sequence. The PCR conditions were: denaturation at 95˚C for 5 min followed by denaturation at 95˚C for 30 sec, annealing at 54˚C for 30 sec and extension at 72˚C for 30 sec for 30 cycles with the last extension step at 72˚C for 7 min. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control. The primers for GAPDH used were sense 5'-TGATGACATCAAGAAGGT GGT-3' and antisense 5'-TCCTTGGAGGCCATGTGGGCC-3'. A 10 μl aliquot from a total of 50 μl PCR reaction mix was subjected to 1.5% agarose gel. The gel was stained with ethedium bromide and destained to visualize PCR products (45). The PCR amplified products were quantified by scanning the ethidium bromide stained bands using Bio-Rad software and normalized against the GAPDH values.

Activation of MAPK cascades and Western blot analysis. LßT2 cells were grown in 6-well plates and starved of serum overnight prior to the experiment, as described above. The cells were pretreated with various inhibitors for 1 h prior to being treated with 10 nM GnRHA for time periods indicated in the figure legends. The cells were washed twice with ice-cold PBS and harvested by scraping into 100 μl of cold lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin] that was added directly into the wells. The lysates were

collected and incubated on ice for 5 min before centrifugation to pellet debris. The protein concentration of clarified supernatant was determined using Bradford assay (Bio-Rad). Protein (40 μg) from each sample was fractionated by 10% SDS-PAGE and electroblotted onto Hybond nitrocellulose (Amersham Biosciences), followed by immunoblotting with rabbit monoclonal anti-phospo ERK (1:1000 dilution), p38 (1:1000 dilution) or JNK (1:1000 dilution) antibody. The blots were incubated overnight at 4˚C with gentle shaking. Total MAPKs were detected with polyclonal antibodies for the various MAPKs as a control for sample loading. Nur77 protein expression in LßT2 was analyzed by incubation with an anti-Nur77 antibody (1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Each blot was stripped and probed with ß-actin antibody (1:5000 dilutions; Sigma) to confirm equal loading for each sample was used as a loading control.

Results and Discussion

GnRH induces expression of Nur77. Recently, members of the Nur77 family have been shown to play a role in the regulation of a number of cellular functions (4,15,46-48). In the brain, Nur77 has been linked to the development and maturation of a specific set of central nervous system neurons and a possible association between the deregulation of Nur77 and neuron degeneration in Parkinson's disease has been reported (49,50). The expression of Nur77 in the pituitary (35) suggests that it may play a role in the regulation of pituitary hormones and genes. However, physiological responses that regulate the expression of Nur77 in pituitary cells including gonadotropes have not been elucidated. In previous studies, we utilized DNA microarray analysis of the effects of GnRHA on LßT2 cells to gain insights into the potential mechanisms by which GnRHA affects mouse pituitary gonadotrope cells and found significant induction of Nur77 (36). Therefore, in the present studies, we first examined the induction of Nur77 in LßT2 cells by semi-quantitative RT/PCR analysis. Consistent with our previous study, using the Nur77 specific primers, PCR generated a 400 bp product that corresponded to the published sequence (Fig. 1A). The increase in expression of Nur77 mRNA was rapid, reaching a maximum level (17-fold) within 2 h and then returning to nearly basal levels after 24 h of treatment. This finding of rapid induction of Nur77 is consistent with reports of its rapid induction in other systems (3,4,37,38). Pretreatment of cells with a GnRH antagonist, Antide (1 mM) completely blocked the effect of GnRHA (Fig. 1B), suggesting that the stimulatory effect of GnRHA is specific and achieved through its high affinity receptors present on cell membranes of LßT2 cells (36). The specificity of the effects is further supported by its dose-dependence, with an IC_{50} value of ~0.05 nM (Fig. 2). Pretreatment of the cells with the transcriptional inhibitor, actinomycin D, blocked the effects of GnRHA on induction of Nur77 mRNA (data not shown). Thus, the changes in the expression of Nur77 mRNA on GnRHA treatment can be attributed to an increase in mRNA biosynthesis, rather than to an alteration in mRNA stability and suggest that the increase in Nur77 mRNA levels induced by GnRHA is due to activation of transcription of the *Nur77* gene.

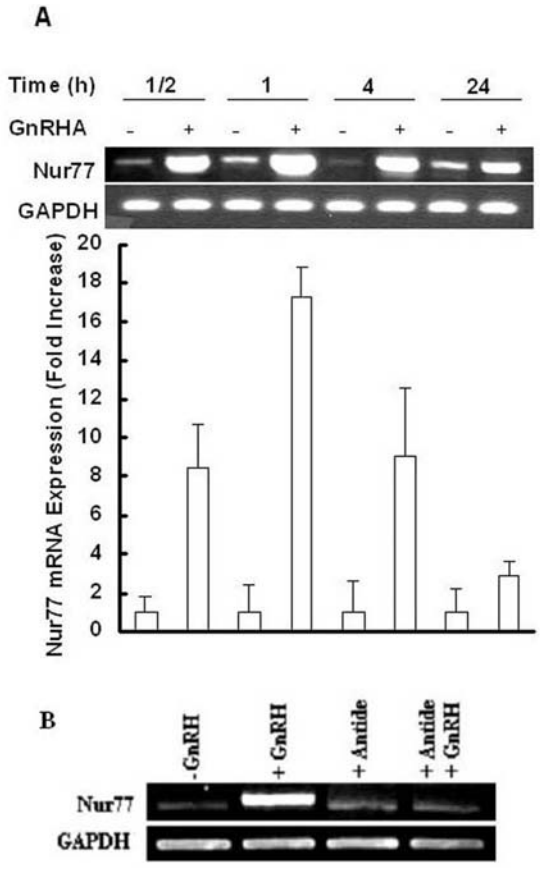

Figure 1. GnRHA induces expression of Nur77 in the mouse pituitary gonadotroph cell line, LßT2. Cells were serum-starved overnight and then treated with 10 nM GnRHA for 1/2, 1, 2, 4 and 24 h (A). To block GnRH receptors, cells were pretreated with 1 μM Antide for 1 h and then with 10 nM GnRHA for 1 h (B). Total RNA (1 μg) was reverse transcribed. Nur77 cDNA was amplified using PCR with specific primers. The expression of GAPDH was used as an internal control. Nur77 mRNA expression was quantified and normalized using the values for the levels of GAPDH mRNA, which was used as an internal control for each sample. The data are representative of three independent experiments with bar graphs displaying the mean ± SEM.

Next, we confirmed the induction of Nur77 at the protein level using Western blot analysis. LßT2 cells treated with vehicle expressed very low levels of an immunoreactive protein with a molecular weight of ~77 kDa. The addition of GnRHA resulted in enhanced expression of Nur77 protein expression, with the levels increasing over time until 12 h (Fig. 3). Taken together our results demonstrate that GnRHA induces Nur77 expression through an effect on transcription and generates a Nur77 protein that corresponds to native Nur77.

In limited studies, regulation of expression of Nur77 by LH in testicular Leydig cells (10) and by LH and hCG in granulose cells of preovulatory follicles (51,52) have been demonstrated, suggesting an important role of Nur77 in the LH-mediated steroidogenesis in Leydig cells and LH-mediated ovulatory process respectively. Similarly, regulation of expression of Nur77 by a variety of stress stimuli in CRH-producing neurons of the hypothalamus (53,54) and by ACTH in adrenal gland leading to enhancement of steroid-21-α-hydroxylase gene transcription (18,55) has been shown. Our finding of induction of Nur77 expression by GnRH in the pituitary gonadotropes, together with its induction by LH in

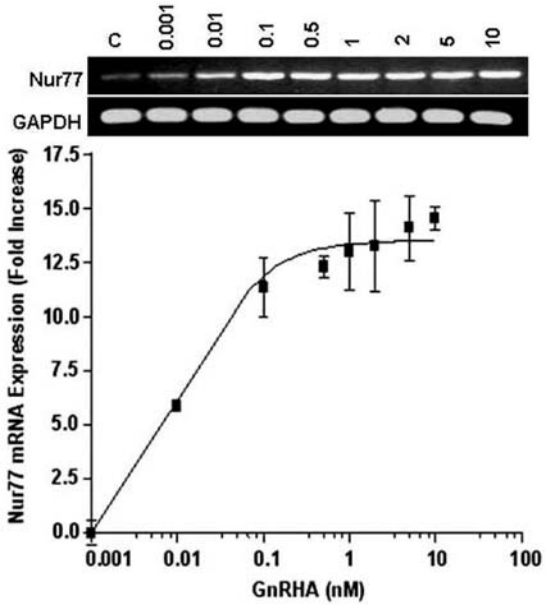

Figure 2. GnRHA induces Nur77 expression in LßT2 cells in a dose-dependent manner. LßT2 cells were serum-starved overnight and then treated with different concentrations of GnRHA for 1 h. Total RNA (1 μg) was reverse transcribed. Nur77 cDNA was amplified using PCR with specific primers. The expression of Nur77 mRNA was normalized using the GAPDH internal control. The data are representative of three independent experiments and are shown as mean ± SEM.

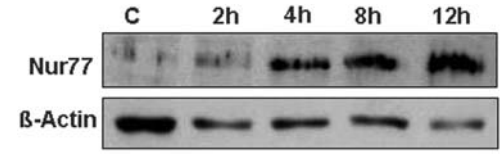

Figure 3. GnRHA induces expression of Nur77 protein in LßT2 cells. LßT2 cells were serum-starved overnight and then treated with 10 nM GnRHA for the indicated time periods. The cells were washed with cold PBS and lysed in lysis buffer. Cell extract (40 μg) from each sample was analyzed by immunoblotting with Nur77 antibody. ß-actin was used as a loading control. The data are representative of two independent experiments.

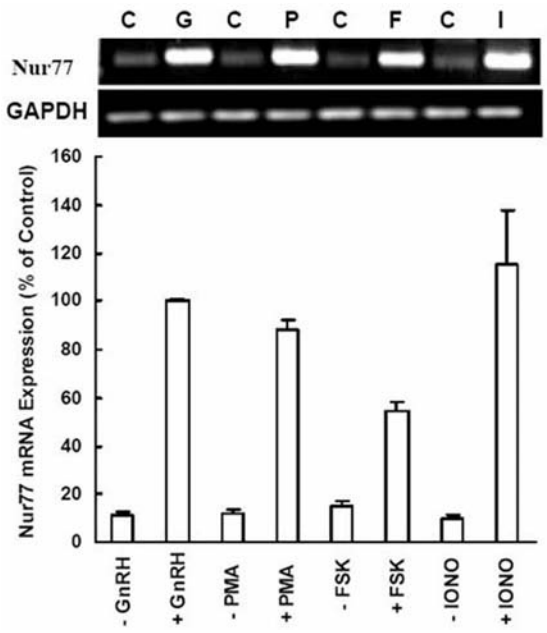

Figure 4. Multiple signaling pathways regulate induction of Nur77 expression by GnRHA. LßT2 cells were serum-starved overnight and then treated with 10 nM GnRHA (G), 1 μM PMA (P), 10 μM forskolin (FSK) (F) or 1 M ionomycin (IONO) (I) for 1 h. Total RNA (1 μg) was reverse transcribed and analyzed using PCR with Nur77 cDNA-specific primers as described above. The expression of GAPDH was used as an internal control. The Nur77 mRNA expression was quantified and normalized using the GAPDH internal controls. The normalized values were then compared with the normalized values for the expression of Nur77 induced by GnRHA, which was arbitrarily assigned a value of 100%. The data are representative of three independent experiments and are shown as the mean ± SEM.

the testicular Leydig cells and preovulatory follicles, suggest that Nur77 may play a dynamic role in the function of the hypothalamic-pituitary-gonadal axis. The physiological consequences of the GnRH effect on Nur77 expression in pituitary gonadotropes have yet to be determined, however.

Similarly, the effects of Nur77 on the downstream signaling pathways of pituitary gonadotropes have yet to be established. The role of Nur77 as a transcription factor is well documented (56,57). Newly synthesized Nur77 protein translocates to nucleus where it binds to NGFI-B (Nur77) binding response elements (NBREs) as a monomer, homodimer, or heterodimer and activates the transcription of various downstream target genes (58). In this respect, Sadie *et al* (59) showed the existence of two nuclear receptor half-sites (NRS) at -244/-236 and -15/-7 (relative to the translation start site) in the flanking sequence of the mouse *GnRH receptor* gene. Both these sites were shown to be overlapping binding sites for Nur77 and SF-1 transcription factor *in vitro*. Expression of Nur77 in the pituitary gonadotrope cell line (αT31) has no effect on basal promoter activity of GnRH receptor, but suppresses the PKA-induced promoter activity in the absence of SF-1 binding site at -15/-7 NRS, suggesting that Nur77 may serve as a negative regulator of PKA-induced *GnRH receptor* gene expression (59).

Multiple signaling pathways are involved in GnRH-mediated induction of Nur77 gene expression in LβT2 cells. GnRH is a hypothalamic decapeptide, which serves as a key regulator of the reproductive system. GnRH signals are transmitted through a specific G-protein coupled receptor, the GnRH receptor, which on activation interacts with the heterotrimeric G_q protein (60,61). This interaction results in recruitment of a diverse group of intracellular signaling cascades, including protein kinase A (PKA), protein kinase C (PKC), G-protein coupled receptor kinases, calcium-calmodulin (Ca^{2+}/CaM), MAPK kinase pathways and increases intracellular calcium levels in pituitary and other cells that express GnRH receptors (reviewed in ref. 43). These signaling cascades culminate mainly in the production and secretion of the LH and FSH.

To determine which GnRHR-coupled signaling pathway mediates the GnRH-induced upregulation of expression of Nur77, we treated LßT2 cells with signaling agonists. Both 10 μM forskolin (FSK) and 1 μM PMA, which activate PKA and PKC pathways respectively, significantly increased the expression of Nur77 mRNA (Fig. 4). Similarly, 1 μM ionomycin, a calcium ionophore increased the expression of Nur77 (Fig. 4). As these results indicated that both the PKA

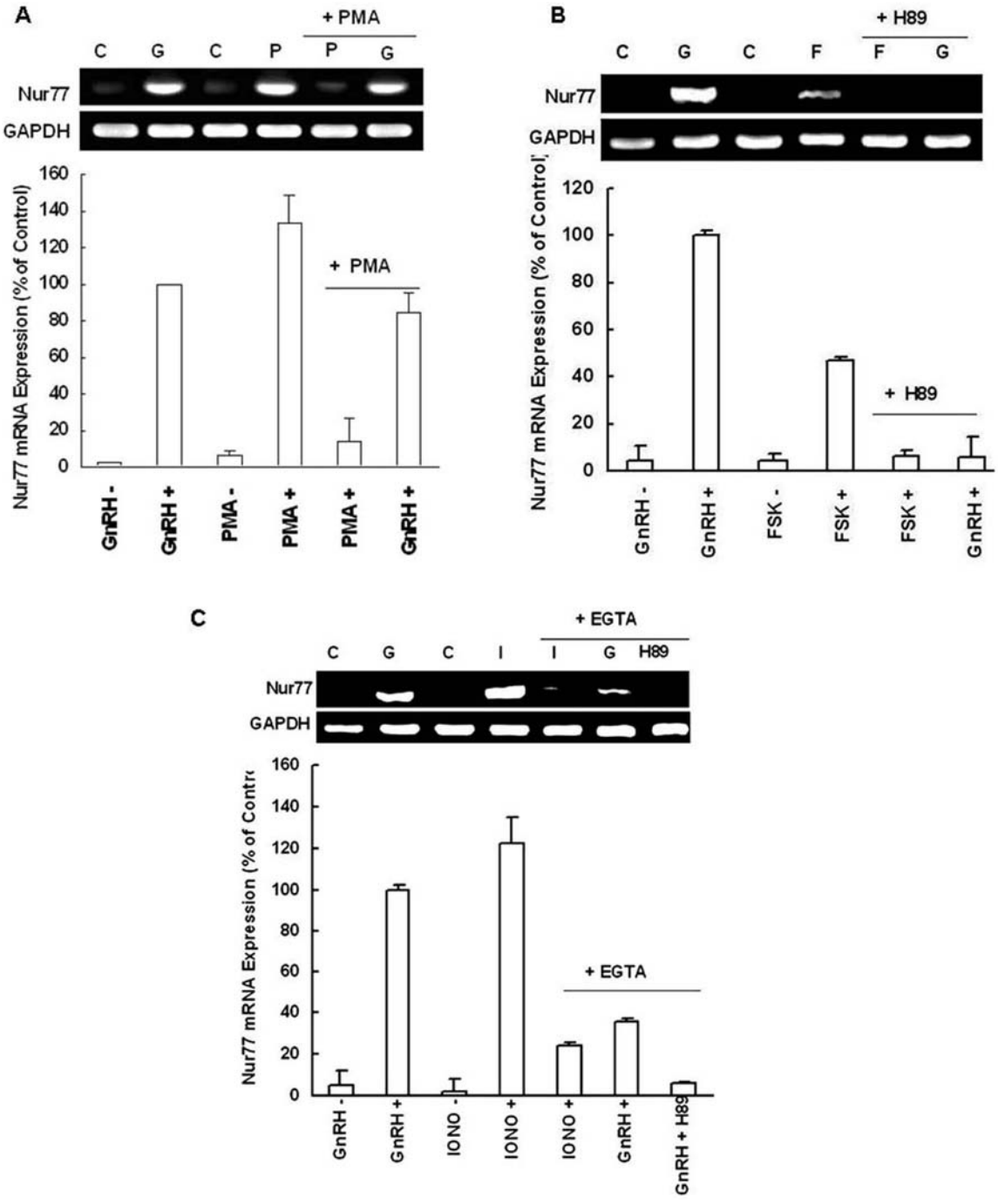

Figure 5. Blockade of the PKA pathway and calcium influx, but not the PKC pathway, inhibits GnRHA-induced Nur77 mRNA expression in LßT2 cells. To deplete PKC, the cells were incubated overnight with 10 μM PMA and then treated with 1 μM PMA (P) or 10 nM GnRHA (G) for 1 h (A). To inhibit the PKA pathway, the cells were pretreated with 30 M H89 for 1 h and then treated with 10 μM FSK (F) or 10 nM GnRHA (G) for 1 h (B). To deplete extracellular calcium, the cells were pretreated with 2.5 mM EGTA for 20 min and then treated with 1 M ionomycin (I) or 10 nM GnRHA (G). To deplete extracellular calcium and to block PKA pathway, cells were treated with 2.5 mM EGTA and 30 μM H89 for 1 h. Total RNA (1 μg) from each sample was reverse transcribed and analyzed for Nur77 mRNA expression using PCR. The expression was normalized using the GAPDH internal control. The normalized values were then compared with the normalized expression of Nur77 on GnRHA induction, which was arbitrarily assigned a value of 100%. Bars shown represent pretreatment. The data are representative of three independent experiments and are shown as the mean ± SEM.

and PKC signaling pathways are involved in the GnRH-mediated induction of Nur77, we tested selective inhibitors of the PKA and PKC pathways for their ability to block induction of Nur77. Pretreatment of cells with 30 μM H-89 (PKA inhibitor) significantly inhibited GnRHA- and FSK-induced expression of Nur77 (Fig 5B). Pretreatment of cells overnight with 1 μM PMA, which depletes PKC, resulted in a complete loss of stimulatory effect of PMA but marginally inhibited the GnRHA-induced expression of Nur77 (Fig. 5A). The addition of 2.5 mM EGTA to the extracellular medium, which chelates calcium, significantly reduced the effects of 1 μM ionomycin and GnRHA on Nur77 mRNA expression (Fig. 5C). Taken together, these results suggest that the effects of GnRHA on the induction of Nur77 are mediated through multiple signaling pathways. These observations concur with the demonstration of the involvement of multiple

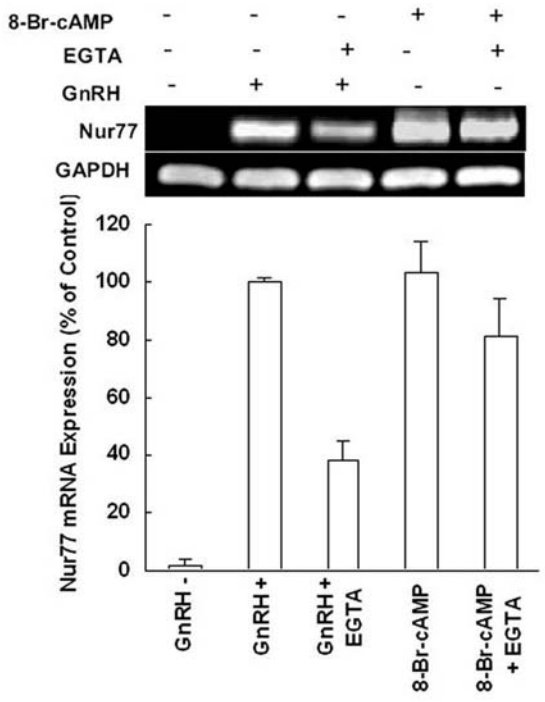

Figure 6. GnRHA induces Nur77 mRNA expression through the activation of the cAMP-PKA signaling pathway and calcium influx. LßT2 cells were serum-starved overnight and then pretreated with 2.5 mM EGTA 20 min followed by treatment with 10 nM GnRHA or 1 mM 8-Br-cAMP for 1 h. Total RNA (1 μg) was reverse transcribed and analyzed for Nur77 mRNA expression using PCR. The expression was quantified and normalized using the GAPDH internal controls. The normalized values were then compared with the normalized expression of Nur77 on GnRHA induction, which was arbitrarily assigned a value of 100%. The data are representative of three independent experiments and are shown as the mean ± SEM.

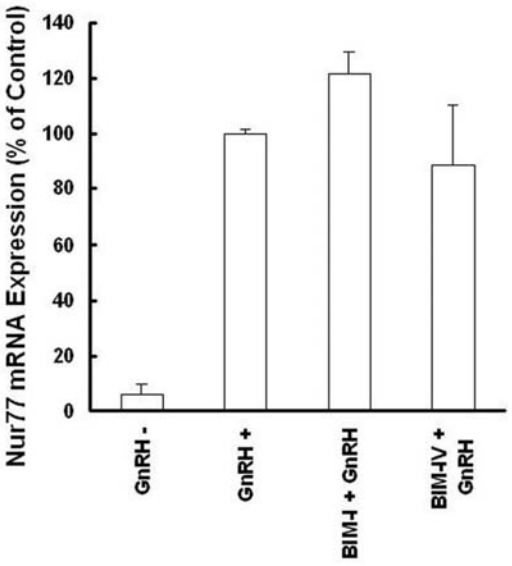

Figure 7. Activation of PKC is not involved in regulation of induction of Nur77 mRNA by GnRHA in LßT2 cells. Cells were serum-starved overnight and then pretreated with PKC-specific inhibitors [BIM-I (1 μM) or BIM-IV (5 μM)] for 1 h followed by treatment with 10 nM GnRHA for 1 h. Total RNA (1 μg) was reverse transcribed and Nur77 mRNA expression was analyzed using PCR with specific primers. Nur77 mRNA expression was quantified and normalized using the GAPDH internal control. The normalized values were then compared with the normalized expression of Nur77 on GnRHA induction, which was arbitrarily assigned a value of 100%. The data are representative of three independent experiments and are shown as the mean ± SEM.

pathways and specifically these pathways in the regulation by GnRH of various GnRH-response genes including LHß, FSHß, Egr1 and Egr2 in pituitary cells (62).

Blockade of PKA and calcium influx, but not PKC inhibits GnRH-induced activation of Nur77 mRNA expression in LßT2 cells. As reported above it appears that GnRHA activation of most, if not all, the major signaling pathways is involved in its ability to regulate the expression of *Nur77* gene in LßT2 cells. To further identify the major pathway, we tested PKA- and PKC-specific inhibitors and extracellular calcium chelator for their ability to block Nur77 mRNA induction by GnRHA. As reported above, treatment of LßT2 cells with 10 μM PMA overnight resulted in a significant (90%) inhibition of PMA-induced Nur77 expression, but showed only a marginal inhibition of GnRHA-induced expression (Fig. 5A), indicating that PKC activation can induce Nur77 mRNA expression, but the ability of GnRHA to upregulate Nur77 does not seem to rely on PKC activation. In contrast, pretreatment of LßT2 cells with the PKA-specific inhibitor, H-89 (30 μM), significantly inhibited both GnRHA-induced and FSK-induced Nur77 mRNA expression (~90%) (Fig. 5B). Treatment of LßT2 cells with 1 mM 8-Br-cAMP, a cell membrane permeable analog of cAMP, was found to significantly increase the expression of Nur77 mRNA (Fig. 6). These results suggest that GnRH induces expression of Nur77 mRNA expression primarily through cAMP/PKA cascade. To rule out the possibility that PKC signaling pathways play a major role in the induction of Nur77 expression in LßT2 cells by GnRH, we analyzed the role of PKC in more detail using PKC-specific inhibitors BIM-I and BIM-IV. As shown in Fig. 7, pretreatment of LßT2 cells with BIM-I (1 μM) or BIM-IV (5 μM) for 1 h prior to the treatment of cells with GnRHA for 1 h did not affect GnRHA-induced Nur77 mRNA expression further indicating that the PKC signaling cascade may not be involved in the induction of Nur77 mRNA by GnRHA in LßT2 cells. Our findings are consistent with the reports of the involvement of activation of cAMP/PKA pathway in the induction of Nur77 transcription by other hormones in other tissues, including corticotropin (ACTH) treatment of adenocortical Y1 cells (18), LH treatment of testicular Leydig cells (10), parathyroid treatment of osteoblastic cells (63) and thyroid stimulating hormone treatment of thyroid cells (64). However, this cAMP-dependence does not seem to be a general phenomenon as the PKC cascade has been reported to be a major pathway involved in Nur77 expression in C2C12 myoblastic cells (65), LS-180 colon adenocarcinoma cells (66) and T cells (67).

To determine the contribution of extracellular calcium to GnRH-induced expression of Nur77 mRNA, we investigated the effect of extracellular calcium chelation on Nur77 mRNA expression. Treatment of LßT2 cells with EGTA (2.5 mM for 20 min) prior to treatment with either 1 μM ionomycin or 10 nM GnRH significantly blocked the effect of both ionomycin (80%) and GnRHA (65%) on Nur77 mRNA expression (Fig. 5C). Pretreatment of LßT2 cells with H-89 (30 μM) plus EGTA (2.5 mM) for 1 h completely blocked the GnRHA-induced expression of Nur77 mRNA (Fig. 5C).

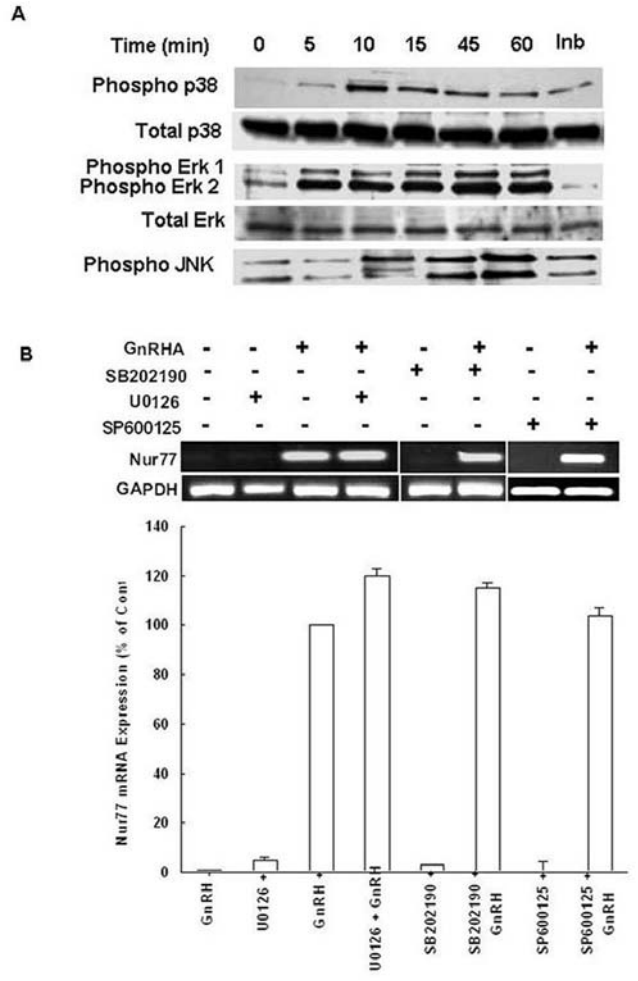

Figure 8. Activation of MAPK signaling pathways are not involved in induction of Nur77 mRNA by GnRHA in LßT2 cells. Cells were serum-starved overnight and then treated with 10 nM GnRHA for the indicated time periods (A). After treatment, the cells were washed with cold PBS and lysed in lysis buffer. Cell lysate (40 μg) from each sample was analyzed on Western blot using the phospho antibodies specific for p38, Erk1/2 or JNK. The intensity of staining of the antibodies reactive with p38 total protein or Erk1/2 total protein was used to confirm equal loading for each sample (A). To inhibit the phosphorylation of p38, Erk1/2 or JNK and to determine Nur77 expression, cells were pretreated with specific inhibitor for p38 (SB202190, 2 μM), Erk1/2 (U0126, 10 μM) or JNK (SP600125, 20 μM) for 30 min followed by treatment with 10 nM GnRHA for 5 min for p38 and for 45 min for both Erk1/2 and JNK. Total RNA (1 μg) was reverse transcribed and analyzed for Nur77 mRNA expression using PCR. GAPDH mRNA expression was used as an internal control. Nur77 mRNA expression was quantified and normalized using the GAPDH internal controls (B). The normalized values were then compared with the normalized expression of Nur77 on GnRHA induction, which was arbitrarily assigned a value of 100%. The data are representative of three independent experiments and are shown as the mean ± SEM.

Pretreatment of cells with EGTA partially (20%) blocked the stimulatory effect of 8-Br-cAMP (Fig. 6), suggesting the importance of calcium influx in activation of Nur77 expression by GnRHA. Calcium has been demonstrated to have multiple modulatory influences on hormone-stimulated adenylate cyclase (68,69) and to play an important in role in pulsatile release of gonadotropin-releasing hormone (70) and in stimulating the effect of GnRH on secretion and expression of LH in gonadotropes (71,72). Since, 8-Br-cAMP directly activates PKA without involving adenylate cyclase, therefore, our results strongly suggest that induction of Nur77 by GnRH is mediated by two independent pathways namely calcium influx and the PKA pathway, but acts predominately through activation of PKA signaling. It remains to be determined if PKA itself or other downstream kinase target Nur77.

Blockade of phosphorylation of p38, Erk1/2 and JNK does not block GnRHA-induced Nur77 expression. PKC activation has been shown to be a major signaling pathway by which GnRH in pituitary and other cells mediates its effects (73,74). As our studies indicate that cAMP/PKA acts as the primary signaling pathway in induction of Nur77 expression by GnRHA in LßT2 cells, we extended our study to the evaluation of molecules that act downstream of PKC, including the analysis of the activation states of Erk1/2, p38 and JNK.

In agreement with earlier published studies (75), treatment of LßT2 cells with GnRHA for various time periods resulted in a time-dependent phosphorylation of p38, Erk1/2 and JNK (Fig. 8). Phosphorylation of p38 occurred within 5 min of treatment, reaching a maximum level within 10 min (Fig. 8A). The maximum levels of phosphorylation of Erk1/2 and JNK were attained within 45 and 60 min of treatment, respectively (Fig. 8A). As expected, pretreatment of cells with p38 MAPK inhibitor SB202190 (2 μM for 30 min), MEK-specific inhibitor U0126 (10 μM for 30 min) or JNK inhibitor SP600125 (20 μM for 45 min) resulted in inhibition of phosphorylation of p38, Erk1/2 and JNK (Fig. 8A). Notably, however, these pretreatments failed to block the GnRHA-induced expression of Nur77 (Fig. 8B). These results strongly suggest that even though GnRH activates PKC signaling in LßT2 cells, this pathway is not involved in the GnRH-mediated induction of Nur77 expression.

Currently, the physiological significance of the induction of Nur77 by GnRHA in pituitary gonadotropes remains unclear. Using DNA microarray analysis technique, we (36) and others (76) have shown changes in expression of a large number of genes in LßT2 cells on treatment with GnRH or GnRHA for 1, 2, 4 and 24 h. Since Nur77 is an early response gene and is known to regulate the expression of other genes, it is plausible that Nur77 serves as a signal that mediates the ability of GnRH to regulate the secretion and expression of pituitary gonadotropins (reviewed in ref. 77). It is possible that some of these functions of GnRH, including secretion and expression of gonadotropins, may be mediated through Nur77. It should be noted, however, that knockout mice have a normal phenotype (19). This may reflect a functional redundancy among Nur77 family members such that stimuli that induce Nur77 can also induce expression of the other family members, such as Nurr1 (10). It is also possible that Nur77 is not involved in the regulation of the secretion and expression of gonadotropins in pituitary, but regulates the extra-pituitary functions of GnRH such as regulation of tumor cell proliferation and apoptosis.

GnRH and its high affinity receptors have been identified in a variety of extra-pituitary tissues including ovary, testis, breast and prostrate (78-82) and various tumors (83-90). Several studies suggest that the GnRH is involved in tumor cell proliferation, apoptosis, cell cycle arrest (41,91-94) and cytoskeletal rearrangements (42). Nur77 has been reported to regulate cell proliferation of prostate, colon, pancreatic and

gastric cancers (9,11,14,15,26). Thus we anticipate that Nur77 may be involved mechanistically in at least some of the functions mediated by GnRH and its high affinity receptors. The molecular mechanism(s) by which Nur77 exerts GnRH function in pituitary and extra-pituitary tissues and antitumor effect of GnRH is an area of active investigation.

In summary, we have shown that GnRH regulates the expression of the orphan nuclear receptor gene, *Nur77*, in the mouse pituitary gonadotrope cell line, LßT2. GnRHA treatment induces Nur77 gene expression through diverse signaling pathway. However, cAMP-PKA signaling pathway appears to be the major signaling pathway involved in GnRH-induced Nur77 expression. Identification of signaling mechanism(s) regulated by Nur77 will be necessary for a full understanding of the role of Nur77 in the GnRH-mediated responses in pituitary, extra-pituitary tissues and tumors.

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