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Elk-1, CREB, and MKP-1 Regulate Egr-1 Expression in Gonadotropin-Releasing Hormone Stimulated Gonadotrophs

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ABSTRACT

Stimulation of gonadotropin-releasing hormone (GnRH) receptors with the GnRH analogue buserelin enhances expression of the zinc finger transcription factor Egr-1 in a pituitary gonadotroph cell line. The signaling cascade is blocked by overexpression of MAP kinase phosphatase-1 that dephosphorylates extracellular signal-regulated protein kinase in the nucleus. Chromatin immunoprecipitation experiments revealed that the phosphorylated form of Elk-1, a key regulator of gene transcription driven by serum response element (SRE), binds to the 5′-upstream region of the Egr-1 gene in buserelin-stimulated gonadotrophs. Expression of a dominant-negative mutant of Elk-1 completely blocked Egr-1 expression, indicating that Elk-1 connects the intracellular signaling cascade elicited by activation of GnRH receptors with transcription of the Egr-1 gene. GnRH receptor activation additionally induced the phosphorylation of CREB, which in its phosphorylated form bound to the Egr-1 gene. Expression of a dominant-negative mutant of CREB reduced GnRH receptor-induced upregulation of Egr-1 expression, indicating that CREB plays a role in the signaling pathway that regulates Egr-1 expression in gonadotrophs. We further identified the genes encoding basic fibroblast growth factor, tumor necrosis factor α, and transforming growth factor β as bona fide target genes of Egr-1 in gonadotrophs. The analysis of gonadotroph cells that express—in addition to GnRH receptors—muscarinic M_3 acetylcholine receptors revealed that the nuclear events connecting GnRH receptors and muscarinic M_3 acetylcholine receptors with the Egr-1 gene are indistinguishable. J. Cell. Biochem. 105: 1267–1278, 2008.

KEY WORDS: Egr-1; Elk-1; GnRH RECEPTOR; M_3 MUSCARINIC ACETYLCHOLINE RECEPTOR; MKP-1; MKP-5

The decapeptide gonadotropin-releasing hormone (GnRH) is the main regulatory factor in the neuroendocrine control of reproduction stimulating the de novo synthesis and secretion of gonadotropins from the anterior pituitary. GnRH is secreted from the hypothalamus into the hypophyseal portal circulation and ultimately binds to plasma membrane-located heptahelical receptors in gonadotrophs in the anterior pituitary gland. The GnRH receptors are preferentially coupled to $G\alpha_{q/11}$ and binding of GnRH therefore activates phosphoinositide and Ca^{2+} signaling and other important signaling molecules including extracellular signal-regulated protein kinase (ERK) [Caunt et al., 2006; Dobkin-Bekman et al., 2006].

Stimulation of GnRH receptors changes the gene expression pattern of pituitary gonadotrophs and activation of the transcription factors Egr-1, c-Jun, and c-Fos has been reported [Halvorson et al., 1999; Tremblay and Drouin, 1999; Wolfe and Call, 1999; Mulvaney and Roberson, 2000; Wurmbach et al., 2001; Duan et al., 2002; Liu et al., 2002; Zhang and Roberson, 2006]. The biosynthesis of the zinc finger protein Egr-1 is regulated by many environmental signals including hormones, growth factors, and neurotransmitters [Thiel and Cibelli, 2002; Rössler et al., 2006]. The importance of Egr-1 in the pituitary is emphasized by the consequences of inactivating the Egr-1 gene in mice. These animals, although viable, have low body mass, are infertile due to defects in hormone regulation and have pituitary glands that are considerably smaller than normal. The anterior lobe in particular is reduced in size and this is associated with a reduction in the number of growth hormone-positive cells. Furthermore the expression of luteinizing hormone (LH) β is

Abbreviations used: ATF, activating transcription factor; CREB, cAMP response element binding protein; Egr, early growth response; ERK, extracellular signal-regulated protein kinase; GnRH, gonadotropin releasing hormone; MKP, MAP kinase phosphatase; PTEN, phosphatase and tensin homolog; SRE, serum response element.

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completely blocked [Lee et al., 1996; Topilko et al., 1997]. Thus, the expression of Egr-1 target genes is critical for the survival and function of cells within the anterior pituitary, including the pituitary gonadotrophs that are central to the control of fertility.

In the present study we have investigated the nuclear mechanisms by which GnRH receptor stimulation is able to activate Egr-1 in immortalised mouse pituitary gonadotroph cells (αT3–1) that express endogenous GnRH receptors [Windle et al., 1990]. Recently, we showed that activation of muscarinic M_3 acetylcholine receptors induces the biosynthesis of Egr-1 in neuroblastoma cells [Rössler et al., 2008]. To compare the regulation of Egr-1 expression via activation of two types of $G\alpha_{q/11}$-coupled receptors, we additionally analyzed αT3–1cells that also expressed muscarinic M_3 acetylcholine receptors (αT3M3) [Willars et al., 1998]. Here, we show that the nuclear events connecting GnRH receptors and M_3 muscarinic receptors with the Egr-1 gene are indistinguishable. These signaling cascades require the activation of MAP kinases, in particular ERK and JNK. We further show that ternary complex factor activation is of major importance in connecting GnRH- and carbachol-induced signaling cascades with enhanced Egr-1 gene transcription. In addition, the basic region leucine zipper transcription factor CREB is phosphorylated following GnRH receptor activation and, using a dominant-negative mutant of CREB, we demonstrate that this transcription factor is involved in connecting receptor stimulation with Egr-1 gene transcription. Finally, we have identified the genes encoding basic fibroblast growth factor, tumor necrosis factor α, and transforming growth factor β as bona fide Egr-1 target genes of Egr-1 in gonadotrophs.

MATERIALS AND METHODS

CELL CULTURE

Murine αT3–1 and αT3M3 cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The medium used to culture the αT3M3 cells also contained 300 μg/ml G418. Cells were incubated for 24 h in medium without serum before stimulation. Stimulation with the GnRH analogue buserelin (Sigma–Aldrich, Steinheim, Germany, # B3303, used at a concentration of 10 nM) or carbachol (Sigma–Aldrich, # C-4382, used at a concentration of 1 mM) was performed as indicated. The MAP kinase kinase inhibitor PD98059 was purchased from Axxora (Lauser, Switzerland, # 385–023), dissolved in DMSO and used at a concentration of 50 μM as suggested [Davies et al., 2000].The cAMP analogue N^6, 2′-*O*-dibutyryladenosine 3′,5′-monophosphate (dbcAMP) was purchased from Sigma–Aldrich (# D0627) and used at a concentration of 1 mM. The phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) was purchased from Sigma–Aldrich (# I5879), dissolved in DMSO and used at a final concentration of 0.5 mM.

LENTIVIRAL GENE TRANSFER

The lentiviral transfer vectors pFUW-REST/Elk-1ΔC and pFUW-MKP-1 have been described elsewhere [Stefano et al., 2006; Bauer et al., 2007]. To generate an expression vector encoding a REST/CREB fusion protein, a *Bgl*II/*Bam*HI fragment of plasmid pGEM-CREB was subcloned into plasmid pCMV-FLAG-REST-N [Day et al., 2004]. The coding region was released with *Ecl*136II and *Bam*HI, filled in using the Klenow fragment of DNA polymerase I and inserted into plasmid pFUW [Lois et al., 2002]. REST/CREB retains the basic region leucine zipper (bZIP) domain of CREB, but lacks the activation domains. The fusion protein additionally contains the N-terminal repression domain of REST, a FLAG epitope and a nuclear localization signal (NLS). Plasmid pFUWMKP-5, a lentiviral transfer vector encoding human MKP-5, contains the MKP-5 cDNA cloned into plasmid pFUW. The lentiviral transfer vector pFWEgr-1.2luc encoding a luciferase reporter gene under the control of 490 nucleotides of the human Egr-1 5′-flanking region has been described elsewhere [Rössler et al., 2008]. The viral particles were produced as previously described [Stefano et al., 2006] by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein and the transfer vector.

REPORTER GENE ANALYSIS

Lysates were prepared using cell culture lysis buffer (Promega, Mannheim, Germany) and luciferase activities were measured [Thiel et al., 2000]. Luciferase activity was normalized to the protein concentration.

WESTERN BLOTS

Nuclear extracts were prepared as described [Kaufmann and Thiel, 2002]. Nuclear proteins (20 μg) were separated by SDS–PAGE and the blots were incubated with antibodies directed against Egr-1 (Santa Cruz, Heidelberg, Germany, # sc-189), Sp1 (Santa Cruz, #sc-59), or the phosphorylated forms of CREB (Upstate Biotechnology, Lake Placid, NY, # 06–519). To detect phosphorylated ERK in the nucleus, 20 μg of proteins derived from nuclear extract preparations were separated on SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with an antibody directed against the phosphorylated form of ERK (Promega, # V8031). Immunoreactive bands were detected using the ECL plus system (Amersham, Braunschweig, Germany). Densitometric analysis of signal intensities was performed by using QuantityOne quantification analysis software (Biorad, München, Germany). Data shown are mean ± SD from three independent experiments. Values were considered significant when $P < 0.05$.

RT–PCR

RT-PCR was performed as previously described [Bauer et al., 2007]. The primers are listed in Table I.

CHROMATIN IMMUNOPRECIPATION

Chromatin immunoprecipation experiments were performed as previously described [Rössler et al., 2008]. The primers are listed in Table I. The antibodies used for chromatin immunoprecipitation were anti di-methyl H3K9 (Abcam, Cambridge, UK, # ab7312), anti tri-methyl H3K4 (Abcam, # ab8580), anti-Egr-1 (Santa Cruz, #sc-110), anti-phospho-CREB (Upstate Biotechnology, # 06–519), and anti-phospho-Elk-1 (Santa Cruz, #sc-8406).

TABLE I. Primers for RT-PCR and ChIP

Genes	Forward primer	Reverse primer	Size of product (bp)	GenBank accession no.
List of gene-specific primers for RT-PCR				
TGFα	gactctccacctgcaagacc	agacagccactcaggcgtat	506	NM_11577.1
bFGF	ggaaacagaggcaggatgaa	gaataagggttgcccagaca	510	AL645982.26
TNFα	ttttccgagggttgaatgag	gaataagggttgcccagaca	456	U68416
PTEN	ggcactgctgtttcacaaga	tctggatttgatggctcctc	501	NM_008960.1
GAPDH	ccctgcatccactggtgctgc	cattgagagcaatgccagccc	292	NM_008084
List of gene-specific primers for ChIP-PCR				
TGFα	gcccacgctaagatgaagac	cctggctgtctggaggat	195	AY340221
bFGF	gcctagcgggacagattctt	gaggggagccccttgagtgta	146	AL645982.26
TNFα	cacacacacctcctgattg	cctcggaaaacttccttggt	204	U68416
PTEN	cccagcctgttgtacctttg	aagagtcccgccacatcac	272	AC060781.8
Egr-1	gtgcccaccactcttggat	cgaatcggcctctatttcaa	157	X12617
Insulin	gaaggtctcaccttctgg	gggggttactggatgcc	271	X04725

RESULTS

BIOSYNTHESIS OF Egr-1 IN BUSERELIN AND CARBACHOL-TREATED GONADOTROPHS

αT3–1 and αT3M3 cells were serum-starved for 24 h and then incubated with the GnRH analogue buserelin. The cells were harvested, nuclear extracts prepared and Egr-1 expression analyzed via immunoblotting. Egr-1 immunoreactivity was almost undetectable in the absence of stimulation and buserelin stimulation significantly increased its levels (Fig. 1A). Similar results were obtained following stimulation of αT3M3 cells with carbachol, an agonist for muscarinic acetylcholine receptors (Fig. 1B). A comparison of the kinetics of buserelin and carbachol-induced increases in the levels of Egr-1 in αT3–1 and αT3M3 cells revealed that peak expression of Egr-1 occuring after 1 h of stimulation.

ELEVATED Egr-1 PROMOTER ACTIVITY IN BUSERELIN AND CARBACHOL-TREATED GONADOTROPHS

We inserted an Egr-1 promoter/luciferase reporter gene into the chromatin of αT3M3 cells using lentiviral gene transfer. The luciferase gene is controlled by 490 nucleotides of the human Egr-1 gene 5′ upstream region. Figure 1C shows a schematic depiction of the integrated provirus encoding the Egr-1 promoter/luciferase reporter gene. αT3M3 cells were infected with recombinant lentiviruses and stimulated with buserelin or carbachol. The addition of buserelin or carbachol strongly induced reporter gene transcription controlled by 490 nucleotides of the human Egr-1 promoter. Luciferase activities were enhanced in buserelin and carbachol-stimulated αT3M3 cells by ≈3.6-fold and ≈3.8-fold, respectively.

EXPRESSION OF MKP-1 IMPAIRS BUSERELIN AND CARBACHOL-INDUCED Egr-1 EXPRESSION

Stimulation of either GnRH receptors or muscarinic M_3 acetylcholine receptors leads to the activation of ERK [Liu et al., 2002; Roberson et al., 2005]. The activated kinase subsequently translocates to the nucleus. MKP-1 is synthesized in different cell types following ERK activation suggesting that MKP-1 is part of a negative feedback loop leading to the inactivation of ERK [Keyse, 1998]. In αT3–1 and LβT2 cells GnRH stimulation induces expression of MKP-1 [Wurmbach et al., 2001; Zhang and Roberson, 2006]. We therefore tested whether overexpression of MKP-1 counteracts the stimulus-induced biosynthesis of Egr-1. Nuclear proteins of mock-infected αT3–1 cells or cells infected with a lentivirus containing the coding information for FLAG-tagged MKP-1 were fractionated by SDS–PAGE. The tagged MKP-1 protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 2A). Using an antibody that detected the phosphorylated form of ERK2 we show that overexpression of MKP-1 abolished the phosphorylation of ERK2 in the nucleus following stimulation with either buserelin or carbachol (Fig. 2B). Accordingly, Egr-1 biosynthesis was almost completely blocked in αT3–1 cells infected with a MKP-1-encoding lentivirus following stimulation with buserelin (Fig. 2C). Similar results were obtained with buserelin and carbachol-stimulated αT3M3 cells that overexpressed MKP-1 (Fig. 2D). In contrast, expression of the transcription factor Sp1 was detectable in mock and virus-infected cells to a similar degree.

Stimulation of GnRH receptors enhances the activity of JNK [Mulvaney and Roberson, 2000; Harris et al., 2003]. To assess the role of JNK in the signaling cascade that connects GnRH receptor stimulation and transcription of the Egr-1 gene, we overexpressed the dual-specific phosphatase MKP-5 that selectively dephosphorylates JNK and p38 protein kinase. MKP-5 does not inactivate ERK [Tanoue et al., 1999]. Figure 2E reveals that Egr-1 expression was reduced in MKP-5 expressing gonadotrophs. However, expression of MKP-1 was much more efficient to block the signaling cascade connecting buserelin or carbachol stimulation with enhanced Egr-1 gene transcription (compare the quantified Egr-1 levels shown in Figure 2C,E). In addition, experiments employing PD98059, a compound that inhibits the phosphorylation of the MAP kinase kinase, thus blocking the activation of ERK [Dudley et al., 1995], revealed that ERK activation is a key event in the signaling cascades that connects GnRH receptor and M_3 muscarinic acetylcholine receptor stimulation with Egr-1 gene transcription (Fig. 2F). The activation of JNK plays a minor role in the regulation of Egr-1 expression in buserelin-stimulated αT3–1 cells.

CHROMATIN IMMUNOPRECIPITATION REVEALS BINDING OF PHOSPHORYLATED Elk-1 TO THE Egr-1 GENE

In the nucleus, phosphorylated ERK is able to change the transcriptional program by phosphorylation of transcriptional regulatory proteins. Phosphorylation of the ternary complex factor

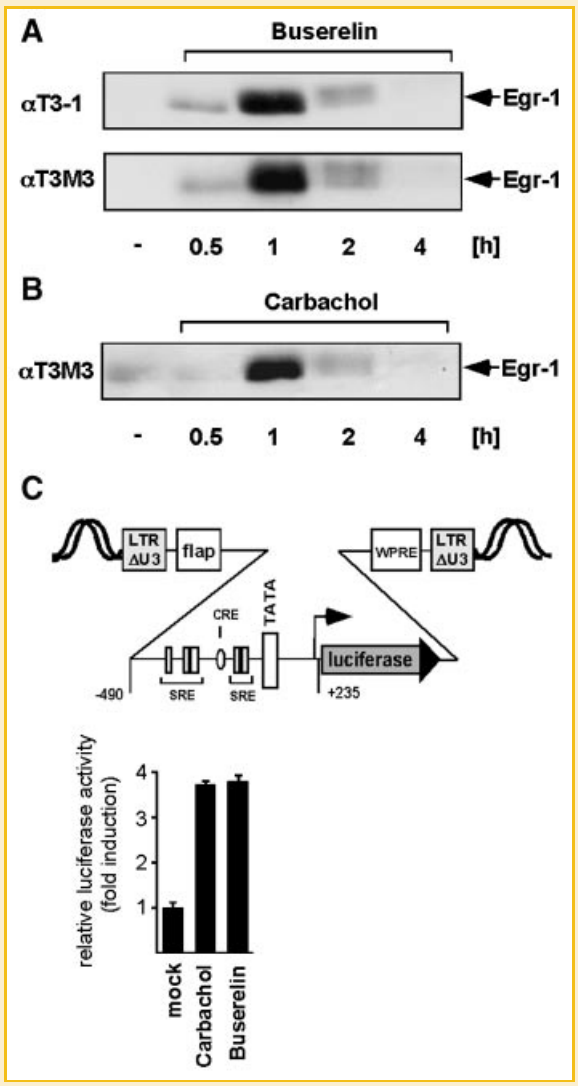


Fig. 1. Expression of Egr-1 in gonadotrophs following GnRH receptor or type M_3 muscarinic acetylcholine receptor stimulation. A: αT3-1 and αT3M3 cells were serum-starved for 24 h and then treated with buserelin (10 nM) for the indicated periods. B: αT3M3 cells were serum-starved for 24 h and then treated with carbachol (1 mM) as indicated. C: Transcription of Egr-1 promoter/luciferase reporter genes in buserelin and carbachol treated αT3M3 gonadotrophs. Schematic representation of the integrated provirus encoding an Egr-1 promoter/luciferase reporter gene. Transcription of the luciferase was controlled by the sequences from −490 to +235 derived from the human Egr-1 gene. The important genetic elements within the Egr-1 regulatory region are shown, including five SREs, and a CRE. αT3M3 cells were infected with a recombinant lentivirus encoding an Egr-1 promoter/luciferase reporter gene. The infected cells were stimulated with buserelin or carbachol as indicated. Cell extracts were prepared and analyzed for luciferase activities, which were normalized to the protein concentrations. Each experiment illustrated here and in all subsequent figures was repeated a minimum of three times with consistent results.

Elk-1 connects the ERK signaling cascade with serum response element (SRE)-mediated transcription. To investigate the existence of a link between phosphorylation of Elk-1 and the upregulation of Egr-1 biosynthesis, we performed chromatin immunoprecipitation experiments. Cross-linked and sheared chromatin prepared from unstimulated αT3-1 cells and αT3-1 cells stimulated with buserelin was immunoprecipitated with phospho-specific antibodies against Elk-1. Figure 3A shows that phosphorylated Elk-1 bound to the regulatory region of the Egr-1 gene when the cells had been stimulated with buserelin. In contrast, we did not detect binding of phosphorylated Elk-1 to the Egr-1 regulatory region in unstimulated cells.

SUPPRESSION OF TERNARY COMPLEX FACTOR ACTIVITY IMPAIRS THE UPREGULATION OF Egr-1 EXPRESSION IN BUSERELIN OR CARBACHOL-STIMULATED GONADOTROPHS

We next tested the impact of ternary complex factor activation on Egr-1 expression using a dominant-negative mutant of the ternary complex factor Elk-1, termed REST/Elk-1ΔC (Fig. 3B). This mutant retains the DNA-binding and SRF interaction domains, but lacks the C-terminal activation domain of Elk-1. REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST [Thiel et al., 1998], a FLAG epitope for immunological detection and a NLS. Nuclear proteins of mock-infected αT3M3 cells or cells infected with a REST/Elk-1ΔC encoding lentivirus were fractionated by SDS–PAGE. The fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope. Figure 3C shows that the REST/Elk-1ΔC fusion protein was synthesized as expected. Furthermore, REST/Elk-1ΔC blocked the biosynthesis of Egr-1 both in buserelin-stimulated αT3-1 cells (Fig. 3D) and αT3M3 cells stimulated with either buserelin or carbachol (Fig. 3E). In contrast, the expression of Sp1 was not changed in mock infected cells in comparison to cells infected with the REST/Elk-1ΔC encoding lentivirus. Stimulus-induced phosphorylation of ERK2 was not changed as a result of REST/Elk-1ΔC expression (data not shown). We conclude that ternary complex factor activation is essential to connect the buserelin and carbachol-induced intracellular signaling cascades with the Egr-1 gene via the SREs within the Egr-1 promoter.

INDUCTION OF Egr-1 BIOSYNTHESIS IN αT3M3 CELLS TREATED WITH dbcAMP AND IBMX

In addition to five SREs the Egr-1 promoter contains a cAMP response element (CRE) encompassing the sequence 5′-TCACGTCA-3′. This motif has one base different to the canonical CRE sequence 5′-TGACGTCA-3′. The functionality of this element has been a matter of controversy in the past. The fact that forskolin, an activator of adenylate cyclase, did not stimulate Egr-1 promoter activity in human U87 glioma cells was used as an argument that the CRE of the Egr-1 promoter does not function as a cAMP-inducible enhancer element [Meyer et al., 2002]. Likewise, forskolin treatment failed to induce expression of Egr-1 in either HEK293 or αT3-1 cells [von der Kammer et al., 1998; Tremblay and Drouin, 1999]. It has therefore been suggested that ATF2 instead of CREB may control Egr-1 gene transcription mediated through p38/stress-activated protein kinases [Rolli et al., 1999]. To investigate the role of CREB in stimulus-induced biosynthesis of Egr-1 in gonadotrophs, we treated αT3M3 cells with the cAMP analogue dbcAMP in the presence of the phosphodiesterase inhibitor IBMX. Western blot analysis showed that this treatment induced a transient expression of Egr-1 (Fig. 4A) with a peak expression occurring after 1 h of stimulation. Thus,

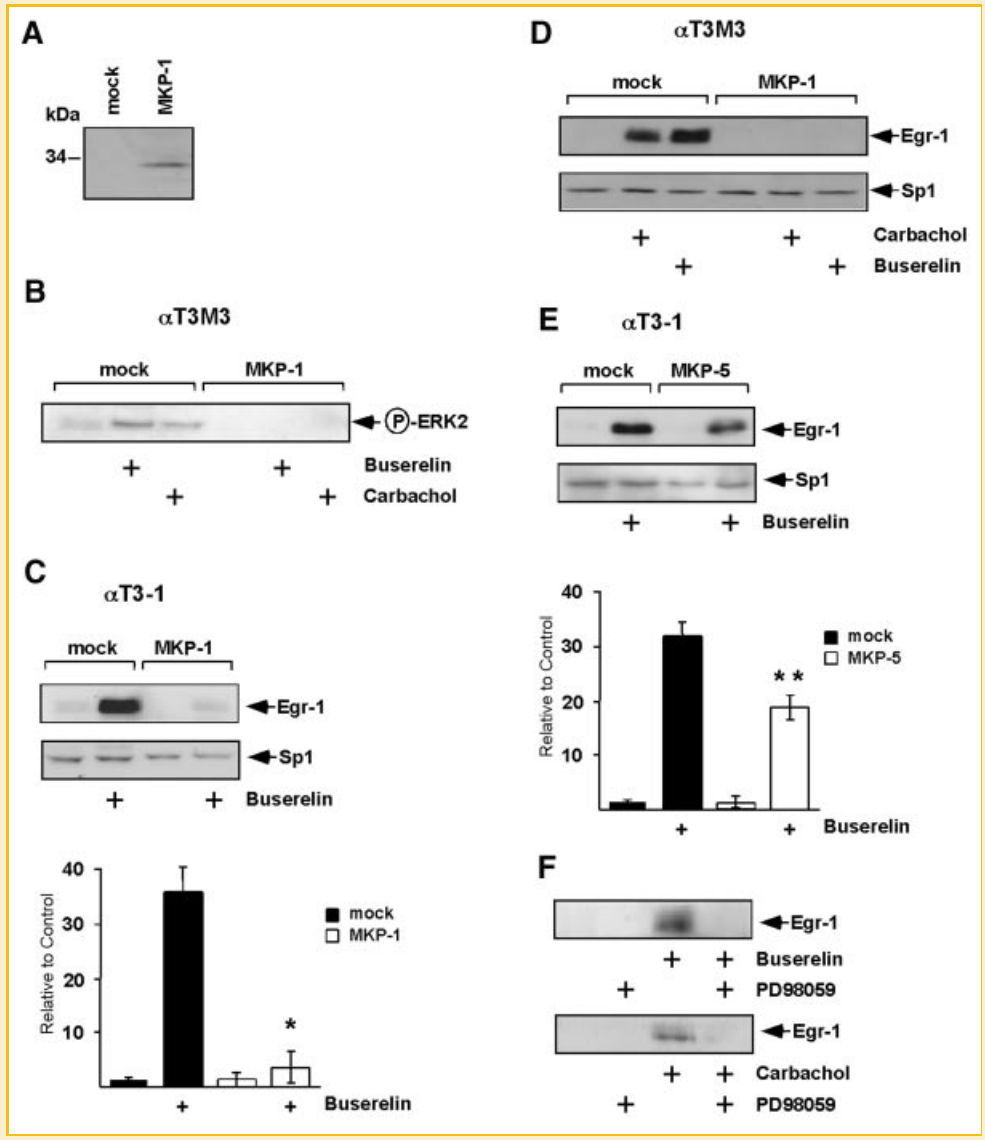


Fig. 2. Role of MAP kinase phosphatases in buserelin and carbachol-induced upregulation of Egr-1 expression in gonadotrophs. A: αT3M3 cells were infected with a recombinant lentivirus encoding FLAG-tagged MKP-1. The transgene was expressed under the control of the human ubiquitin-C promoter. As a control mock-infected cells were analyzed. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against the FLAG epitope. B: Overexpression of MKP-1 blocks phosphorylation of ERK2 in the nucleus. αT3M3 cells were stimulated with buserelin (10 nM) for 10 min or with carbachol (1 mM) for 5 min. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against the phosphorylated form of ERK2. C,D: Overexpression of MKP-1 blocks buserelin and carbachol-induced upregulation of Egr-1 expression. αT3-1 cells (C) or αT3M3 cells (D) were infected with a recombinant lentivirus encoding FLAG-tagged MKP-1. As a control mock-infected cells were analyzed. The cells were serum-starved for 24 h and then treated with buserelin (10 nM) or carbachol (1 mM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. As a control, the expression of Sp1 was assessed in mock and virus infected cells. $^{*}P < 0.005$ (E) αT3-1 cells were infected with recombinant lentiviruses encoding MKP-5. The transgenes were expressed under the control of the human ubiquitin-C promoter. As a control mock-infected cells were analyzed. The cells were serum-starved for 24 h and then treated with buserelin (10 nM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against Egr-1. As a control, the expression of Sp1 was assessed in mock and virus infected cells. $^{**}P < 0.05$ (F) The effect of the MAP kinase kinase inhibitor PD98059 on the biosynthesis of Egr-1 was investigated in aT3M3 cells that had been preincubated for 1 h with PD98059 before stimulation with buserelin or carbachol.

Egr-1 gene expression can be induced in gonadotrophs by elevated cAMP levels. The phosphorylated form of CREB is already detectable 30 min after stimulation. Thus, from the kinetics it seems likely that CREB is phosphorylated and activated first. The phosphorylated from of CREB then transactivates the Egr-1 gene leading to the enhanced transcription of the Egr-1 gene. As a loading control, we also analyzed the expression of Sp1.

BUSERELIN-INDUCED PHOSPHORYLATION OF CREB REQUIRES ERK

Phosphorylation of CREB has been reported in GnRH-stimulated hypothalamic GT1-7 cells [Neithardt et al., 2006]. Figure 4B shows that both buserelin and carbachol induced the phosphorylation of CREB in αT3M3 cells. As a loading control, the expression of Sp1 is depicted.

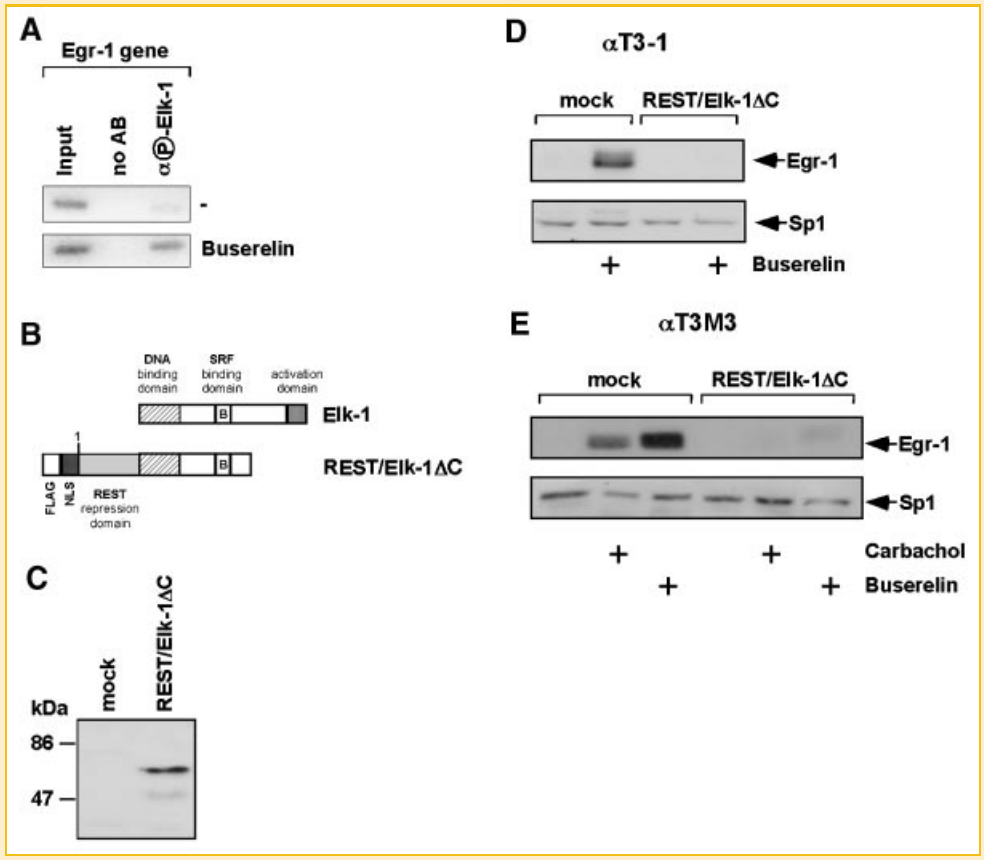
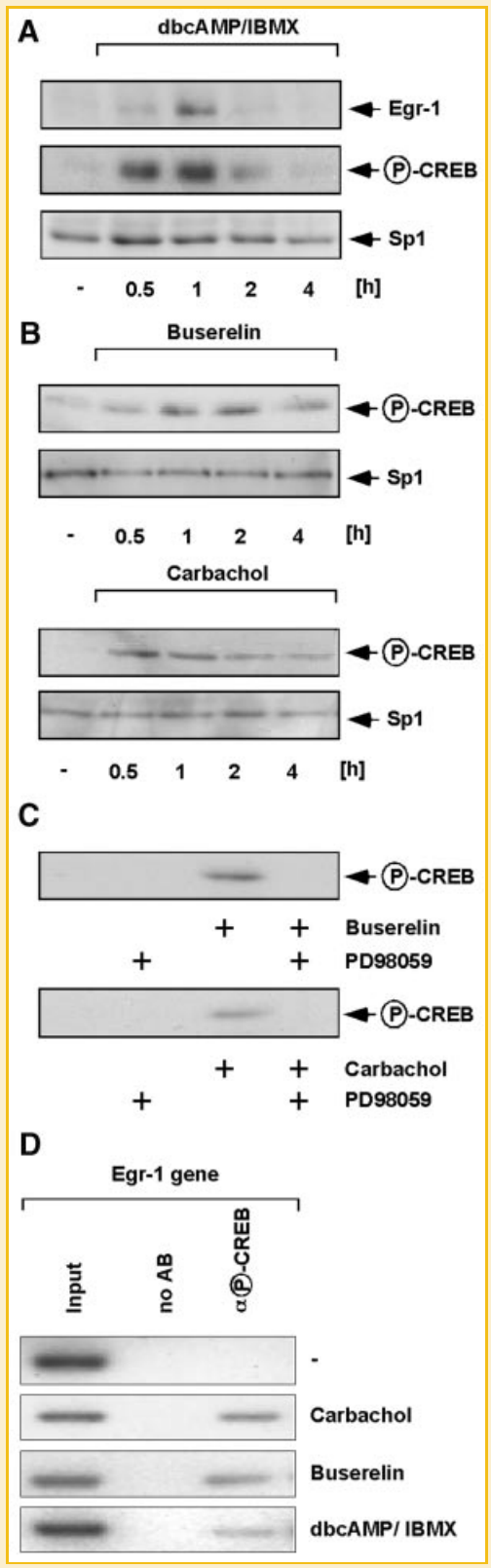


Fig. 3. The phosphorylated form of Elk-1 binds to the Egr-1 promoter and is required to connect buserelin and carbachol signaling with increased biosynthesis of Egr-1 (A) Chromatin immunoprecipitation experiments were performed with chromatin isolated from αT3-1 cells that had been stimulated with buserelin (10 nM, 20 min). As a control, chromatin of unstimulated cells was analyzed. Cross-linked and sheared chromatin was immunoprecipitated with an antibody directed against the phosphorylated forms of Elk-1. B: Schematic representation of wild-type Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. The DNA binding domain is localized on the N-terminus. The transcriptional activation domain is localized on the C-terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phosphoacceptor sites S383 and S389. Elk-1 binds with its B-domain to SRF, allowing the formation of the ternary Elk-1-SRF complex. The B-domain also couples the C-terminal phosphorylation of Elk-1 with enhanced DNA binding via the Ets domain. The dominant-negative mutant REST/Elk-1ΔC lacks the phosphorylation-regulated activation domain, but retains the DNA and SRF binding domains. The truncated Elk-1 is expressed as a fusion protein together with a transcriptional repression domain derived from the transcriptional repressor REST. C: Western blot analysis of αT3M3 cells either mock infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. Western blots were probed with an antibody against the FLAG-tag. Molecular-mass markers in kDa are shown on the left. D,E: Expression of REST/Elk-1ΔC blocks buserelin and carbachol-induced upregulation of Egr-1. αT3-1 (D) and αT3M3 (E) cells were either mock infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. Cells were stimulated with either buserelin (10 nM) or carbachol (1 mM) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. As a control, the expression of Sp1 was determined in mock and virus infected cells.

Stimulation of the GnRH receptor in αT3-1 cells does not produce detectable amounts of cAMP [Horn et al., 1991; Lariuvière et al., 2007]. Thus, an elevation of the intracellular cAMP concentration is not implicated in buserelin-induced upregulation of Egr-1 expression in αT3-1 cells. Activation of ERK may also lead to the phosphorylation of CREB via the mitogen- and stress-activated kinases MSK1 and 2 [Deak et al., 1998; Wiggin et al., 2002]. To directly prove that activation of the ERK signaling pathway induces CREB phosphorylation in αT3-1 cells we pretreated the cells with PD98059. Figure 4C shows that blocking of the ERK signaling pathway abolished the phosphorylation of CREB in buserelin or carbachol-treated αT3-1 gonadotrophs.

CHROMATIN IMMUNOPRECIPITATION REVEALS BINDING OF PHOSPHORYLATED CREB TO THE Egr-1 GENE

To investigate the existence of a link between the phosphorylation of CREB and the upregulation of Egr-1 biosynthesis, we performed chromatin immunoprecipitation experiments. Cross-linked and sheared chromatin prepared from unstimulated αT3M3 cells and αT3M3 cells stimulated with either buserelin, carbachol, or dbcAMP/IBMX was immunoprecipitated with phospho-specific antibodies against CREB. Figure 4D shows that phosphorylated CREB bound to the regulatory region of the Egr-1 gene when the cells had been stimulated with either buserelin, carbachol, or



dbcAMP/IBMX. No binding of phospho-CREB to the Egr-1 gene was observed in unstimulated αT3M3 cells.

SUPPRESSION OF CREB REDUCED THE EXPRESSION OF Egr-1 IN EITHER BUSERELIN OR CARBACHOL-STIMULATED GONADOTROPHS

We directly assessed the involvement of CREB in the signaling cascade connecting activated GnRH receptors or muscarinic M_3 acetylcholine receptors to enhanced Egr-1 biosynthesis in a loss-of-function experiment. We expressed a dominant-negative mutant of CREB termed REST/CREB (Fig. 5A). Nuclear proteins of mock-infected αT3M3 cells or cells infected with a REST/CREB encoding lentivirus were fractionated by SDS–PAGE. The fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope. Figure 5B shows that the REST/CREB fusion protein was synthesized as expected. Next, we assessed the functional effects of REST/CREB expression upon the stimulus-induced biosynthesis of Egr-1. The results show that REST/CREB reduced the expression of Egr-1 in buserelin-stimulated αT3–1 cells (Fig. 5C) and in buserelin and carbachol-stimulated αT3M3 cells (Fig. 5D). Quantification of the Egr-1 levels of three independent experiments revealed that expression of REST/CREB induced a reduction of 30 or 22% in buserelin or carbachol-treated gonadotrophs, respectively. As a control, we stimulated the cells with dbcAMP/IBMX. Here, expression of REST/CREB completely blocked the dbcAMP/IBMX-induced upregulation of Egr-1 (Fig. 5E). We conclude that CREB connects activated GnRH receptors and muscarinic M_3 acetylcholine receptors with enhanced Egr-1 gene transcription. Together, these data indicate that both CREB and Elk-1 are essential in buserelin and carbachol-stimulated gonadotrophs to induce Egr-1 expression.

Fig. 4. CREB is phosphorylated in buserelin- and carbachol-treated gonadotrophs and binds to the Egr-1 gene under physiological conditions (A) αT3M3 cells were incubated with dbcAMP/IBMX (1 mM/0.5 mM). Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against Egr-1 and the phosphorylated form of CREB. As a control, the expression of Sp1 was analyzed. B: αT3M3 cells were serum-starved for 24 h and then treated with either buserelin (10 nM) or carbachol (1 mM). Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against the phosphorylated form of CREB. As a control, the blot was probed with an antibody directed against Sp1. C: The effect of the MAP kinase kinase inhibitor PD98059 on the phosphorylation of CREB was investigated in αT3–1 cells that had been preincubated for 1 h with PD98059 (50 μM) before stimulation with buserelin. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against the phosphorylated form of CREB. D: Chromatin immunoprecipitation experiments were performed with chromatin isolated from αT3M3 cells that had been stimulated with either carbachol (1 mM), buserelin (10 nM), or dbcAMP/IBMX (1 mM/0.5 mM) for 1 h. As a control, chromatin of unstimulated cells was also analyzed. Cross-linked and sheared chromatin was immunoprecipitated with an antibody directed against the phosphorylated form of CREB. Immunoprecipitated chromatin fragments were amplified with primers encompassing the CRE motif of the Egr-1 regulatory region. As a negative control, chromatin immunoprecipitation was performed with protein A-sepharose but without antibodies (no Ab). As a positive control, an aliquot of the total chromatin in the absence of immunoprecipitation was analyzed by PCR (input).

EPIGENETIC CONFIGURATION OF PUTATIVE Egr-1 TARGET GENES IN GONADOTROPHS

To show that the newly synthesized Egr-1 protein is biologically active, we analyzed the expression of Egr-1 target genes. The genes encoding basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), and phosphatase and tensin homolog (PTEN) have been identified as bona fide Egr-1 target genes in other cellular systems. We first analyzed the epigenetic configuration of these genes in gonadotrophs using epigenetic markers that differentiate between actively transcribed or silenced genes. The amino termini of H3 histones can be methylated on lysine residue 9 by methyltransferases such as SUV39H1 or G9a [Rea et al., 2000]. The H3mK9 modification functions as a high affinity binding site for the silencing protein, heterochromatin protein 1 (HP1). HP1 homo- and hetero-dimerizes and promotes the formation of a high-order structure that leads to an extension of the heterochromatic region into neighboring chromatin [Thiel et al., 2004]. Thus, methylation of H3K9 functions as an epigenetic marker for silenced genes. An epigenetic marker for actively transcribed genes is methylation of lysine residue 4 of histone 3 [Santos-Rosa et al., 2002]. We recently confirmed that methylation of lysine residues 3 or 9 of histone 3 correlates very well with either gene expression or gene silencing [Hohl and Thiel, 2005]. We analyzed the methylation status of Egr-1 target genes in gonadotrophs by chromatin immunoprecipitation using antibodies directed against either the tri-methylated form of histone H3K4 or the di-methylated form of histone H3K9. The results show that the Egr-1 binding sites of the bFGF, TNFα, TGFβ, and PTEN genes in gonadotrophs are embedded into a nucleosomal context with histone H3 molecules carrying tri-methylated lysine 4, which is a marker of actively transcribed genes. In contrast, the insulin gene, analyzed as a control, is embedded into a nucleosomal structure characterized by methylation of lysine residue 9 of histone 3 (Fig. 6A).

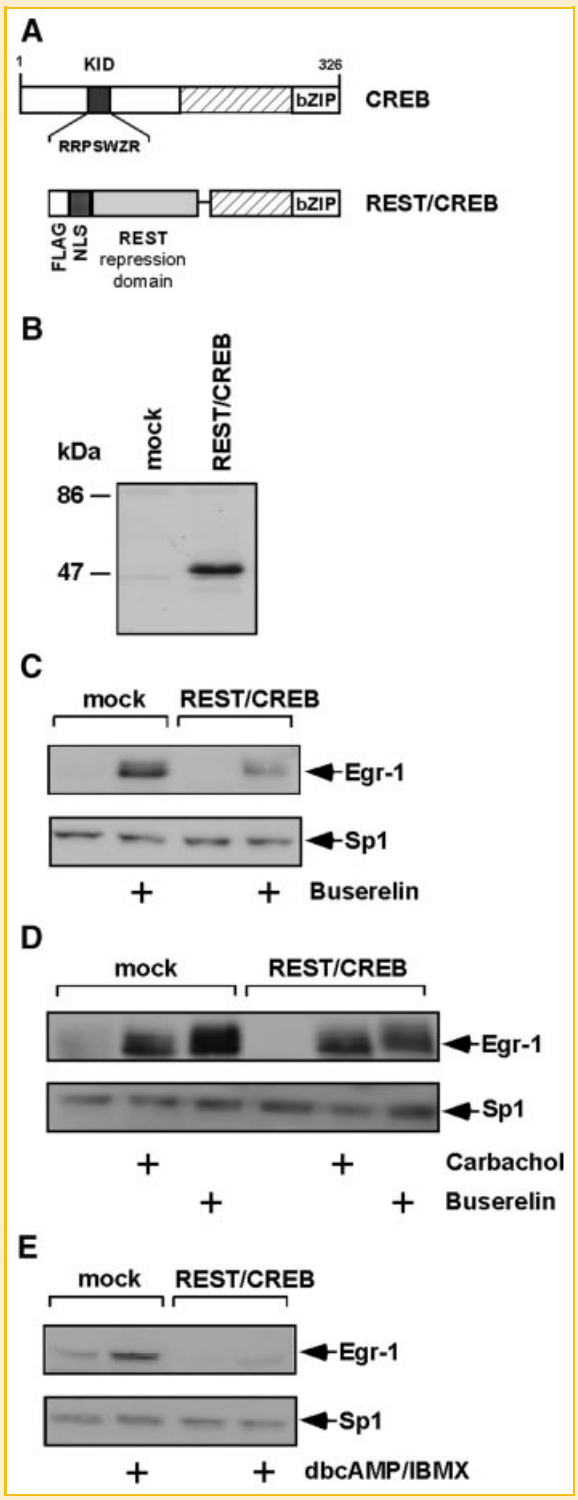
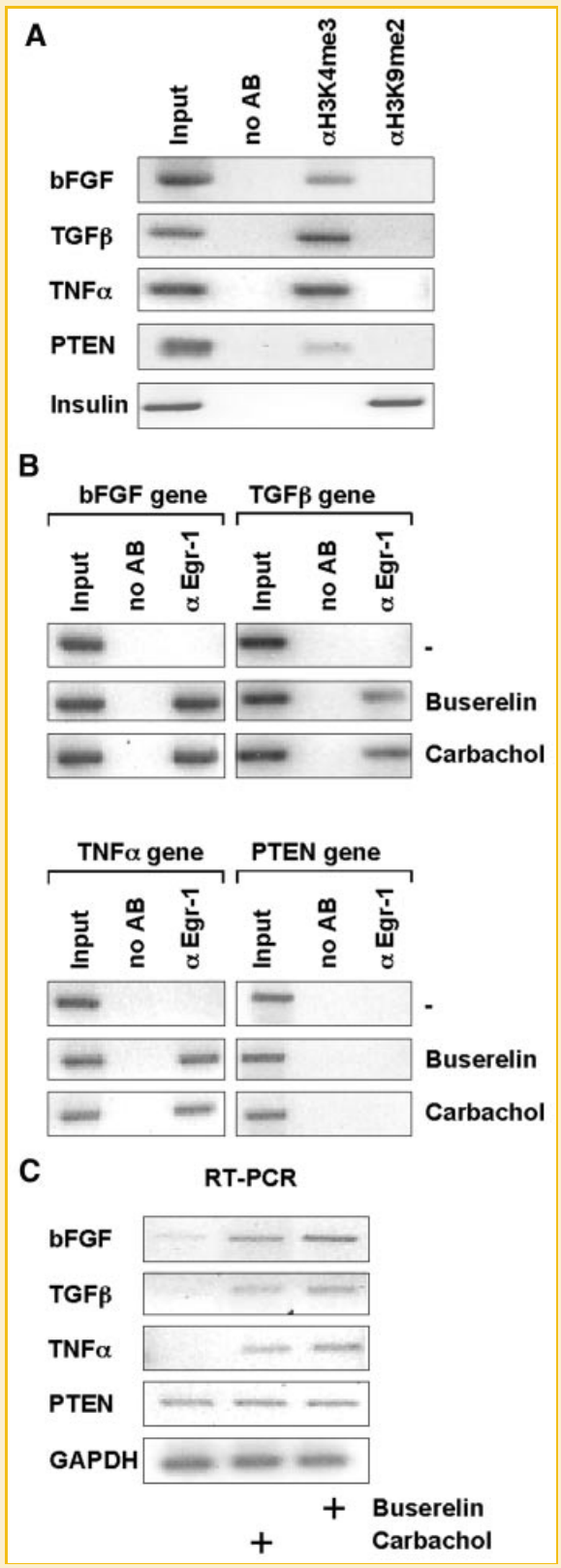


Fig. 5. Expression of a dominant-negative mutant of CREB reduces Egr-1 expression in buserelin and carbachol-stimulated gonadotrophs. A: Schematic representation of wild-type CREB and the dominant-negative mutant REST/CREB highlighting the bZIP domain. The phosphorylation-dependent transcriptional activation domain of CREB (kinase-inducible domain; KID) is also shown as well as the sequence encompassing serine residue 133 that is phosphorylated by protein kinase A. The dominant-negative mutant REST/CREB lacks this KID domain, but retains the DNA and dimerization domains. The truncated CREB molecule is expressed as a fusion protein together with a transcriptional repression domain of REST. B: Western blot analysis of αT3M3 cells infected with a recombinant lentivirus encoding REST/CREB. As a control, extracts from mock transfected αT3M3 cells were analyzed. Western blots were probed with an antibody against the FLAG-tag. Molecular-mass markers in kDa are shown on the left. C,D: Expression of REST/CREB reduces buserelin or carbachol-induced upregulation of Egr-1. αT3-1 (C) and αT3M3 (D) cells were either mock infected or infected with a recombinant lentivirus encoding REST/CREB. Cells were stimulated with either buserelin (10 nM) or carbachol (1 mM). Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. As a control, the expression of Sp1 in mock and virus infected cells was determined (E) REST/CREB interferes with the upregulation of Egr-1 expression induced by dbcAMP/IBMX. αT3M3 cells were either mock infected or infected with a recombinant lentivirus encoding REST/CREB. Cells were stimulated with dbcAMP/IBMX (1 mM/0.5 mM) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. As a control, the expression of Sp1 was determined in mock and virus infected cells.



CHROMATIN IMMUNOPRECIPITATION REVEALS BINDING OF Egr-1 TO THE REGULATORY REGIONS OF THE bFGF, TNFα, AND TGFβ GENES

Using chromatin immunoprecipitation we tested whether Egr-1 binds in a stimulus-dependent manner to the genes encoding bFGF, TNFα, TGFβ, or PTEN. Cross-linked and sheared chromatin prepared from unstimulated αT3M3 cells and αT3M3 cells stimulated with either buserelin or carbachol was immunoprecipitated with an antibody directed against Egr-1. Figure 6B shows that Egr-1 bound to the regulatory region of the bFGF, TNFα, and TGFβ genes, but only when the cells had been stimulated with buserelin or carbachol. In contrast, we were unable to precipitate DNA encompassing the regulatory region of the PTEN gene, indicating that PTEN gene transcription is not controlled by Egr-1 in gonadotrophs. We further measured the bFGF, TNFα, TGFβ, and PTEN mRNA concentration using semi-quantitative RT-PCR analysis. Figure 6C shows elevated bFGF, TNFα, and TGFβ mRNA levels in αT3M3 cells that had been treated with buserelin or carbachol. In contrast, no changes of PTEN mRNA levels were detected between stimulated and non-stimulated αT3M3 cells. Thus, the RT-PCR experiments mirror the data obtained by chromatin immunoprecipitation experiments, revealing that bFGF, TNFα, and TGFβ gene expression, but not PTEN transcription, are controlled by Egr-1 in gonadotrophs.

DISCUSSION

The gene expression pattern of a cell is controlled by extracellular stimuli and a dominant theme in the study of the regulation of gene expression has been the investigation of the mechanisms of selective gene transcription following cellular stimulation. The objective of this study was to compare two signaling cascades in gonadotrophs, initiated by stimulation of either GnRH receptors or muscarinic M_3 acetylcholine receptors, which both couple to $G\alpha_{q/11}$. A number of signaling molecules that are required to connect GnRH receptor stimulation with enhanced transcription of the Egr-1 gene in

Fig. 6. Identification of Egr-1 target genes in gonadotrophs. A: Epigenetic modifications of putative Egr-1 target genes in αT3M3 cells. Chromatin immunoprecipitations were performed with anti-dimethylated H3K9 or anti-trimethylated H3K4 antibodies. Immunoprecipitated chromatin fragments were amplified with primers encompassing the Egr-1 binding sites within the regulatory regions of the genes encoding bFGF, TNFα, TGFβ, and PTEN. In addtion, the epigenetic signature of the insulin gene was assessed. As a negative control, no primary antibody was added (no Ab). An aliquot of the total chromatin was also examined by PCR (input). B: Binding of Egr-1 to the regulatory region of Egr-1 target genes under physiological conditions. Cross-linked and sheared chromatin prepared from unstimulated αT3M3 cells and αT3M3 cells stimulated with either buserelin (10 nM) or carbachol (1 mM) was immunoprecipitated with an antibody directed against Egr-1. Immunoprecipitated chromatin fragments were amplified with primers encompasing the Egr-1 binding sites within the regulatory regions of the genes encoding bFGF, TNFα, TGFβ, and PTEN. As a negative control, no primary antibody was added (no Ab). An aliquot of the total chromatin was also examined by PCR (input). C: Upregulation of TNFα, TGFβ, and bFGF mRNA concentrations in αT3M3 cells as a result of buserelin or carbachol stimulation. αT3M3 cells were stimulated with buserelin for 2 h or with carbachol for 1 h. Total RNA was isolated, the mRNA reverse transcribed and the cDNA analyzed by PCR using primers to detect TNFα, TGFβ, bFGF, and PTEN mRNA.

gonadotrophs have been identified previously [Tremblay and Drouin, 1999; Wolfe and Call, 1999; Duan et al., 2002]. However, the description of the signaling pathway is far from complete and we provide here several new links, focusing particularly on events within the nuclear compartment. In addition, we have identified Egr-1 target genes that are expressed following buserelin stimulation of GnRH receptors or carbachol stimulation of muscarinic M_3 acetylcholine receptors.

GnRH stimulation has been shown previously to stimulate Egr-1 gene transcription in αT3 cells [Halvorson et al., 1999; Tremblay and Drouin, 1999; Duan et al., 2002; Roberson et al., 2005]. Activation of muscarinic acetylcholine receptors has been shown to induce Egr-1 biosynthesis in a number of different cell types [von der Kammer et al., 1998; Rössler et al., 2008]. Our study shows that stimulation of $G\alpha_{q/11}$-coupled muscarinic M_3 receptors triggers the same intracellular signaling pathway as stimulation of GnRH receptors to control transcription of the Egr-1 gene. We have shown that overexpression of MKP-1 leads to dephosphorylation and inactivation of ERK in the nucleus. Pharmacological experiments supported the conclusion that activation of ERK is crucial to connect stimulation of either GnRH receptors or muscarinic M_3 acetylcholine receptors with Egr-1 gene transcription.

Stimulation of GnRH receptors enhances the activity of JNK [Mulvaney and Roberson, 2000; Harris et al., 2003]. Likewise, treatment of the cells with anisomycin, a well characterized activator of the stress regulated protein kinases JNK and p38, induced the biosynthesis of Egr-1 in gonadotrophs (S.I. Mayer, G. Thiel, unpublished observations). Overexpression experiments of MKP-5, a phosphatase that dephosphorylates and inactivates JNK and p38, reduced, but did not abolish buserelin-induced expression of Egr-1. These data indicate that activated JNK is involved in the regulation of Egr-1 transcription. However, JNK plays a minor role in comparison to ERK. The transcription factor ATF2 is a well-known substrate for JNK and p38. ATF2 is phosphorylated as a result of GnRH receptor stimulation and the phosphorylated form of ATF2 binds to the Egr-1 gene in ChIP experiments (S.I. Mayer, G. Thiel, unpublished observations). Thus, JNK may regulate Egr-1 expression via phosphorylation and activation of ATF2. This signaling pathway is blocked following expression of MKP-5.

In the nucleus, phosphorylated ERK is able to change the transcriptional program by phosphorylating transcriptional regulatory proteins. Phosphorylation of the ternary complex factor Elk-1 connects the ERK signaling cascade with SRE-mediated transcription. ERK may also stimulate the transcriptional activity of CREB and ATF1 [Deak et al., 1998; Gupta and Prywes, 2002; Wiggin et al., 2002]. In fact, GnRH has been reported to induce the phosphorylation of both Elk-1 [Duan et al., 2002] and CREB [Neithardt et al., 2006]. We performed a series of experiments to elucidate the role of Elk-1, and CREB in connecting either buserelin or carbachol stimulation with an upregulation of Egr-1 gene transcription. The ternary complex factor Elk-1 contacts DNA and also has to bind to the serum response factor (SRF) to exhibit biological activity. The transcriptional activity of Elk-1 depends on its phosphorylation-status. Elk-1 is phosphorylated by several protein kinases including JNK and ERK, leading to enhanced DNA binding, ternary complex formation and SRE-mediated transcription [Marais et al., 1993]. The Egr-1 promoter contains five SREs encompassing the consensus sequence $CC[A/T]_6GG$. In addition, multiple binding sites for ternary complex factors (Ets) are adjacent to these sequences having the Ets consensus core sequence GGAA/T. Transcriptional activation of Egr-1 is often preceded by an activation of Elk-1, indicating that the SREs within the Egr-1 promoter mediate signal-induced activation of Egr-1 gene transcription. For example, in stimulated glutamatergic corticostriatal neurons a strict spatiotemporal connection between Elk-1 activation and Egr-1 mRNA synthesis has been demonstrated [Sgambato et al., 1998]. It has been shown previously that GnRH triggers the phosphorylation of Elk-1 in αT3–1 cells [Duan et al., 2002]. Here, we have shown directly that the phosphorylated form of Elk-1 bound to the regulatory region of the Egr-1 gene in buserelin-stimulated αT3–1 cells under physiological conditions. Furthermore, we have examined the necessity of ternary complex factor activation for buserelin and carbachol-mediated upregulation of Egr-1 using a dominant negative version of Elk-1 in loss-of-function experiments. Expression of this Elk-1 mutant revealed that ternary complex factor activation is a key step that connects GnRH receptor- and muscarinic M_3 acetylcholine receptor-stimulation with enhanced Egr-1 biosynthesis.

In a series of experiments we have elucidated the role of CREB in the buserelin and carbachol-initiated signaling cascades. We have shown that administration of dbcAMP/IBMX induced the biosynthesis of Egr-1 via activation of cAMP-dependent protein kinase. These results confirm previous data showing that a constitutively active mutant of CREB transactivate an Egr-1 promoter/luciferase reporter gene [Al Sarraj et al., 2005; Bauer et al., 2005]. Furthermore, we have shown that CREB is phosphorylated in buserelin- and carbachol-treated αT3M3 cells and that binding of the phosphorylated form of CREB to the Egr-1 gene occurred in stimulated cells. Expression of a dominant-negative form of CREB reduced the expression of Egr-1 in cells stimulated with either buserelin or carbachol, indicating that phosphorylation and activation of CREB is part of the signaling cascade that connects GnRH receptor and muscarinc M_3 acetylcholine receptor stimulation with enhanced Egr-1 gene transcription.

Previously, knockout experiments revealed that the LH β gene is a target for Egr-1 in the pituitary [Lee et al., 1996; Topilko et al., 1997]. Here, we have identified further Egr-1 targets in αT3M3 cells. A combination of chromatin immunoprecipitation experiments and RT-PCR revealed that the genes encoding bFGF, TNFα, and TGFβ are bona fide target genes of Egr-1 in this gonadotrophs cell line. These genes are also regulated by Egr-1 in other cell types [Biesiada et al., 1996; Liu et al., 1999; Svaren et al., 2000; Fu et al., 2003; Lee et al., 2005]. However, Egr-1 did not transactivate the PTEN tumor suppressor gene in gonadotrophs, as described previously [Virolle et al., 2001]. The PTEN gene is not silenced in gonadotrophs, as revealed by the methylation of lysine residue 4 of histone H3. These data shed light on the fact that Egr-1 target genes are cell-type dependent.

In summary, we have shown that stimulation of GnRH receptors and muscarinic M_3 acetylcholine receptors in gonadotrophs promotes the biosynthesis of Egr-1 and that these different $G\alpha_{q/11}$-coupled receptors use an identical signaling cascade. In

the nucleus, the ternary complex factor Elk-1 connects ERK signaling with the Egr-1 gene via SREs of the Egr-1 promoter. The bZIP transcription factor CREB is phosphorylated in buserelin treated cells, leading to a transactivation of the Egr-1 gene. The stimulus-induced upregulation of MKP-1 functions as a shut-off-device for both buserelin and carbachol-induced signaling cascades. A combination of chromatin immunoprecipitation experiments and RT-PCR revealed that the genes encoding bFGF, TNFα, and TGFβ are bona fide target genes of Egr-1 in gonadotrophs.

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