Molecular and Cellular Endocrinology ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Original article

cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells

Mercedes Mori Sequeiros Garcia [a], Alejandra Gorostizaga [a], Laura Brion [b], Silvia I. González-Calvar [c,d], Cristina Paz [a,*]

[a] *Institute for Biomedical Research (INBIOMED), Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, (C1121ABG), Buenos Aires, Argentina*
[b] *Membrane Signaling Networks, Department of Medicine, Karolinska Institutet, CMM, Karolinska University Hospital-Solna, Solnavägen 1, 171 77 Solna, Stockholm, Sweden*
[c] *Institute of Biology and Experimental Medicine, National Council for Scientific and Technical Research, Vuelta de Obligado 2490 (C1428DN), Buenos Aires, Argentina*
[d] *School of Medicine, University of Buenos Aires, Paraguay 2155 (C1121ABG), Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 6 January 2015
Accepted 26 January 2015
Available online

Keywords:
MAP kinase phosphatase
MKP-1
ERK1/2
Nr4a1
Nur77
Leydig cells

ABSTRACT

In Leydig cells, LH and cAMP promote ERK1/2 activation and MAPK phosphatase-1 (MKP-1) induction. MKP-1 up-regulation, which involves post-translational modifications such as ERK1/2-mediated phosphorylation, reduces ERK1/2 phosphorylation as well as Steroidogenic Acute Regulatory (StAR) protein expression and steroidogenesis. As LH- and cAMP-promoted StAR transcription requires the induction of Nur77, product of *Nr4a1* gene, we analyzed the roles of ERK1/2 and MKP-1 in 8Br–cAMP-mediated *Nr4a1* expression in MA-10 Leydig cells. Pharmacological blockade of ERK1/2 activation partially reduced the 8Br–cAMP-mediated increase in both *Nr4a1* messenger levels and promoter activity. MKP-1 knockdown increased 8Br–cAMP-induced promoter activity, while its over-expression produced the opposite effect. It is concluded that *Nr4a1* induction is dependent on ERK1/2 and that MKP-1 negatively regulates this induction. Experiments based on the over-expression of MKP-1 mutated forms revealed that MKP-1 half life is determined by post-translational modifications in ERK-consensus sites, a regulation that modulates the effect of MKP-1 on *Nr4a1* expression.

1. Introduction

Mitogen-activated protein kinases (MAPK), such as ERKs, JNKs and the members of the p38 subgroup, are involved in several processes like proliferation, differentiation, apoptosis and even steroidogenesis activation (Brion et al., 2011; Cagnol and Chambard, 2010; Raman et al., 2007). As MAPK activation is dependent on phosphorylation in specific threonine and tyrosine residues, the magnitude and duration of MAPK activity are linked to the action of phosphatases capable of dephosphorylating and inactivating them. MAPK phosphatases (MKPs) are a family of dual specificity (threonine and tyrosine) protein phosphatases specifically involved in MAPK regulation (Boutros et al., 2008; Caunt and Keyse, 2013). Through their ability to modulate MAPK, MKPs also modulate all MAPK-dependent processes. Moreover, it has been proposed that the stimuli promoting MAPK activation also promote MKP regulation, which leads to the control of MAPK activity in a precise temporal frame.

MKP-1, a member of the MKP family, is a nucleus-localized phosphatase rapidly induced by different types of stimuli such as hormones (Bey et al., 2003), growth factors (Tong and Hamel, 2007) and stress conditions (Gorostizaga et al., 2013). In adrenocortical and Leydig cells, the adrenocorticotropic hormone (ACTH) and the luteinizing hormone (LH), respectively, promote not only ERK1/2 activation but also MKP-1 induction, which contributes to ERK1/2 dephosphorylation (Bey et al., 2003; Brion et al., 2011). We have demonstrated that, in MA-10 Leydig cells, 8Br–cAMP and LH receptor (LHR) activation by human chorionic gonadotropin (hCG) up-regulate MKP-1 by transcriptional and post-translational mechanisms which include MKP-1 phosphorylation mediated by ERK1/2 (Brion et al., 2011). In addition, we have shown that MKP-1 expression

Abbreviations: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; MEK, kinases upstream ERK1/2; MKP, MAPK phosphatase; PKA, protein kinase A; PKC, protein kinase C; RT, reverse transcription; SF1, steroidogenic factor 1; shRNA, short hairpin RNA; StAR protein, Steroidogenic Acute Regulatory protein; WT, wild type.

* Corresponding author. Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 5th Floor (C1121ABG), Buenos Aires, Argentina. Tel.: +541145083672 ext. 36; fax: +541145083672 ext. 31.
E-mail address: crispaz1506@gmail.com (C. Paz).

http://dx.doi.org/10.1016/j.mce.2015.01.041
0303-7207/

reduces the effect of 8Br–cAMP and hCG on steroidogenesis (Brion et al., 2011).

It is well documented that S359 and S364 are consensus sites for ERK1/2 phosphorylation within the MKP-1 sequence, and that phosphorylation on these residues impairs MKP-1 proteosomal degradation (Brondello et al., 1999). MKP-1 phosphorylation on sites related with its instability is less widely described, although ERK1/2 activation by serum is known to promote MKP-1 phosphorylation on S296 and S323, two additional ERK1/2 phosphorylation consensus sites, which in turn induces protein ubiquitination and degradation (Lin and Yang, 2006).

The post-translational regulation of MKP-1 in cAMP-stimulated MA-10 Leydig cells is dependent on ERK1/2 activity and leads to an increase in MKP-1 protein levels through a mechanism which might involve, at least in part, S359 and S364 phosphorylation. However, the role played by multi-site ERK phosphorylation in MKP-1 half life in c-AMP-stimulated Leydig cells remains elusive. Moreover, it is unknown whether ERK-mediated MKP-1 phosphorylation is relevant to its actions on steroidogenesis.

We have further demonstrated that MKP-1 induction by cAMP or LH in MA-10 Leydig promotes ERK1/2 dephosphorylation and reduces the actions of these stimuli on ERK1/2-dependent events involved in steroidogenesis. LH regulates Leydig cell function through a mechanism that involves protein kinase A (PKA) activation (Podesta et al., 1976), and PKA-dependent ERK1/2 activation (Hirakawa and Ascoli, 2003). The rate-limiting step in steroid biosynthesis, i.e. the delivery of cholesterol from the outer to the inner mitochondrial membrane, is facilitated by the steroidogenic acute regulatory (StAR) protein (Clark et al., 1994; Stocco and Clark, 1996). LH regulates steroidogenesis through the induction of StAR gene (Clark et al., 1994; Stocco and Clark, 1996) and the activation of StAR protein (Arakane et al., 1997; Poderoso et al., 2008), two events dependent on PKA (Arakane et al., 1997; Clark et al., 1994) and on ERK1/2 activity (Gyles et al., 2001; Poderoso et al., 2008). StAR gene transcriptional regulation requires transcription factors already present in the cell which are activated by post-translational modifications, such as Steroidogenic Factor 1 (SF1) and others which must be de novo synthesized, e.g. the Nur77 (which is encoded by *Nr4a1* gene) (Martin et al., 2008). ERK1/2 is known to participate in LH-/cAMP-induced activation of SF1 by phosphorylation (Gyles et al., 2001); in contrast, its role in LH-/cAMP-induced expression of the *Nr4a1* gene in Leydig cells has been scarcely analyzed, even though ERK1/2 mediates *Nr4a1* induction in other systems (Bliss et al., 2012; Stocco et al., 2002).

By means of MKP-1 over-expression and knock-down experiments, we have shown that MKP-1 reduces the stimulatory effect of 8Br–cAMP and hCG on ERK1/2 phosphorylation, StAR gene and protein expression and steroidogenesis (Brion et al., 2011). Thus, MKP-1 could exert its regulatory action on StAR gene by reducing the expression and/or activity of ERK-dependent transcription factors.

According to these observations, this study explores the role played by ERK1/2 in cAMP-stimulated *Nr4a1* expression in MA-10 Leydig cells and the impact of MKP-1 and its post-translational modifications on this regulation.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against β-tubulin, Nur77 and FLAG M2 were purchased from Millipore Corporation (Billerica, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA), respectively; polyclonal antibodies against phospho-ERK1/2 (pERK1/2) and total ERK1/2 were purchased from New England Biolabs, Inc. (Beverly, MA, USA); horseradish peroxidase-conjugated goat-anti-rabbit, goat-anti-mouse secondary antibodies, as well as immuno-blot polyvinylidene fluoride membrane were purchased from Bio-Rad Laboratories (Hercules, CA, USA); 2-(2-amino-3-methoxyphenyl) 4*H*-1-benzopyran-4-one (PD98059) and 8-bromo–cAMP (8Br–cAMP) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell cultures

The MA-10 cell line, a clonal strain of mouse Leydig tumor cells generously provided by Dr. Mario Ascoli (University of Iowa, College of Medicine, Iowa City, IO, USA), was handled as originally described (Ascoli, 1981). After 24 h of serum starvation, the cells were incubated with 8Br–cAMP or other agents.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using Tri Reagent following the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH, USA). Reverse transcription was done using 2 μg of total RNA as previously described (Castilla et al., 2008) and real-time PCR was performed as previously described (Brion et al., 2011) using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA). Reactions were carried out using the SYBR Green Master Mix reagent kit (Applied Biosystems, Carlsbad, CA, USA) and the following specific primers: for *Nr4a1*, forward 5′-GGCTTCTTCAAGCGCACAGT-3′ and reverse 5′-GCTGCTTGGGTTTT GAAGGTAG-3′; for GAPDH cDNA, forward 5′-TGCACCACCAACTGC TTAGC-3′ and reverse 5′-GCATGGACTGTGGTCATGAG-3′. Cycling conditions for *Nr4a1*, step 1: 95 °C 10 min, step 2: 95 °C 15 s, step 3: 62.5 °C 1 min (steps 2 and 3 were repeated 40 times). Cycling conditions for GAPDH, step 1: 95 °C 10 min, step 2: 95 °C 15 s, step 3: 60 °C 1 min (steps 2 and 3 were repeated 40 times). Assessment of quantitative differences between samples in the cDNA target was performed as previously described (Brion et al., 2011).

2.4. Plasmid constructs

pGL3-NR4A1 was generously provided by Takashi Yazawa, Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Fukui, Japan (Inaoka et al., 2008). pFLAG-MKP-1, pSUPER.retro.MKP-1 and pFLAG S359A-S364A-MKP-1 were constructed as previously described (Brion et al., 2011). pSUPER.retro.MKP-1 encodes for a shRNA-MKP-1 which efficiently abrogates endogenous MKP-1 expression in MA-Leydig cells (Brion et al., 2011).

The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was employed to replace serine 296 and 323 with alanine in the pFLAG-MKP-1 sequence to generate the flag-S296A-S323A-MKP-1 double mutant. First, pFLAG-S296A-MKP-1 was obtained using the following oligonucleotides: sense 5′-GAGGC GGAGTATCATCGCCCCGAACTTCAGC-3′ and antisense 5′-GCTGAAGTT CGGGGCGATGATACTCCGCCTC-3′. Then, pFLAG-S296A-MKP-1 was amplified with the specific oligonucleotides to mutate serine 323: sense 5′-CTCTGCTGAAGCTGGGGCCCCTGCCATGGCTGTC-3′ and antisense 5′-GACAGCCATGGCAGGGGCCCCAGCTTCAGCAGAG-3′ (mutated nucleotides are underlined).

2.5. Transfection assays

Cells were seeded the day before transfection, grown up to 80% confluence and transfected during 6 h using Lipofectamine 2000 reagent in Opti-MEM medium according to the manufacturer's instructions (Invitrogen, Life Technologies, Grand Island, NY, USA).

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

2.6. Western blot and immunofluorescence analysis

Proteins were subjected to SDS–PAGE (10%) and electrotransferred onto polyvinylidene fluoride membranes as previously described (Brion et al., 2011). Immunoblotting was performed using the following antibody dilutions: mouse monoclonal anti-FLAG (1:10,000), mouse monoclonal anti-β-tubulin (1:5000), rabbit polyclonal anti-pERK1/2 (1:5000), rabbit polyclonal anti-total ERK1/2 (1:5000), rabbit polyclonal anti-Nur77 (1:2000). Bound antibodies were developed by incubation with secondary antibody (goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated) and detected using the enhanced chemiluminescence detection reagent (GE Life Sciences, Princeton, NJ, USA). Secondary antibodies were used in the following concentrations: goat anti-rabbit horseradish peroxidase-conjugated: 1:10,000 (for pERK and total ERK detection) or 1:5000 (for Nur77 detection); goat anti-mouse horseradish peroxidase-conjugated 1:20,000 (for flag-MKP-1 detection) or 1:5000 (for β-tubulin detection).

For immunofluorescence analysis, MA-10 cells were grown on poly-L-lysine-coated glass coverslips as previously described (Brion et al., 2011) and incubated, following the respective treatments, with anti-Nur77 (1:300), in a humidified chamber for 24 h at 4 °C. Primary antibody was detected by cy2-conjugated goat anti-(rabbit IgG) Ig (1:300). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The glass coverslips were mounted in FluorSave reagent (Calbiochem, San Diego, CA, USA) and examined in an epifluorescence microscope (Nikon Eclipse E200).

2.7. Luciferase assay

For luciferase assays, transfections were carried out in 24-well plates using 0.6 μg pSUPER.retro.MKP-1, pFLAG-MKP1 wild type (WT), pFLAG-S359A-S364A-MKP-1, pFLAG-S296A-S323A-MKP-1 or the empty vector (Mock) with 0.2 μg pGL-3 *Nr4a1*, and 10 ng pRL *Renilla* luciferase expression vector as an internal control for transfection efficiency. *Nr4a1* promoter activity was evaluated in cells treated with 0.5 mM 8Br–cAMP for 4 h to achieve submaximal stimulation. Luciferase activity was measured using the Dual Luciferase Assay System (Promega Corp., Madison, WI, USA) and the Synergy HT luminometer (BioTek Instruments, Inc., Winooski, VT, USA).

2.8. Pulse and chase assays

MA-10 Leydig cells were transfected with the corresponding plasmid, serum-starved for 24 hours the next day and then stimulated with 0.5 mM 8Br–cAMP. After 30 min of stimulation, 2 μg/ml cycloheximide (CHX) was added to the culture media. Cells were processed at different times after CHX treatment, and total protein was subjected to immunoblotting using FLAG antibody and β-tubulin antibody after stripping. After protein detection, the integrated optical density of corresponding band was quantitated by densitometry and the values of flag-MKP-1 (WT or mutated forms) were normalized against the corresponding loading control. The relative levels of flag-MKP-1 (WT or its mutated forms) were expressed as a percentage, taking the value registered after 30 min of CHX treatment (time 0) as 100%.

2.9. Statistical analysis

Unless otherwise indicated, results are shown as the mean ± SEM from three independent experiments, each performed by triplicate. Statistical significance was evaluated using ANOVA followed by Tukey's test. Differences were deemed significant when $P < 0.05$.

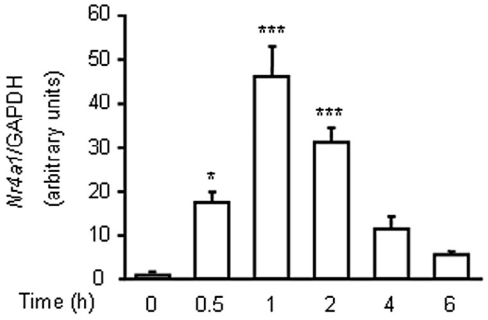

Fig. 1. Effect of cAMP on *Nr4a1* mRNA levels in MA-10 Leydig cells. Cells were serum-starved for 24 h and then incubated with 0.5 mM 8Br–cAMP for the times indicated. Total RNA was isolated and subjected to reverse transcription and real-time PCR using specific primers for *Nr4a1* and GAPDH as loading control. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments. * $P < 0.05$ and *** $P < 0.001$ vs. time 0 h, by ANOVA followed by Tukey's test.

3. Results

A series of experiments were conducted to determine the role of ERK1/2 in the expression of *Nr4a1* triggered by cAMP. It has already been demonstrated that, in MA-10 Leydig cells, 0.5 mM 8Br–cAMP increases *Nr4a1* mRNA levels, which peak after 2 h (~40-fold) (Martin et al., 2009). In this context, we evaluated the time and magnitude of maximal induction of *Nr4a1* in our system, i.e. serum-starved MA-10 Leydig cells. *Nr4a1* mRNA levels were significantly raised after 0.5 h, peaked at 1 h (~40-fold) and returned to basal levels at 4 h (Fig. 1).

In order to determine whether ERK1/2 participates in cAMP-mediated *Nr4a1* induction, mRNA levels and promoter activity were evaluated in cells treated with PD98059, a compound that inhibits ERK-upstream kinase (MEK) and prevents its activation. In serum-starved MA-10 Leydig cells, 8Br–cAMP increased *Nr4a1* promoter activity, causing nearly 19-fold and 20-fold increases after 4 h and 6 h, respectively (data not shown). Therefore, the effect of PD98059 on *Nr4a1* promoter activity was evaluated in cells stimulated for 4 h, while the effect on mRNA levels was evaluated after 1 h of stimulation, i.e. at the time of maximal induction. PD98059 exposure reduced the effect of 8Br–cAMP on mRNA levels and promoter activity (~50% inhibition on both parameters) (Fig. 2A and B). Western blot analyses revealed that 50 μM PD98059 reduced pERK to basal levels, while 10 μM of this inhibitor rendered no significant changes (Fig. 2C).

Nur77 protein levels from control and 8Br–cAMP-stimulated MA-10 cells were also analyzed. Western blot showed a significant increase in this protein after 4 h of stimulation, an effect which was attenuated by 50 μM PD98059 (Fig. 3A). Immunofluorescence analyses showed the accumulation of Nur77 in the nucleus of 8Br–cAMP-stimulated cells, while this accumulation was reduced in the presence of 50 μM PD98059 (Fig. 3B). In addition, Western blot analyses were performed in Y1 adrenocortical cells, an alternative murine steroidogenic cell line. These studies revealed that Nur77 protein levels were increased by 8Br–cAMP stimulation and that this effect was reduced by 50 μM PD98059 incubation (Fig. 3C).

Considering that ERK1/2 inhibition reduced *Nr4a1* promoter activation by cAMP, and the fact that MKP-1 participates in early ERK1/2 dephosphorylation, we performed luciferase assays to evaluate whether this phosphatase could reduce the effect of cAMP on promoter activity. Cells were co-transfected with pGL-3 *Nr4a1*, pRL Renilla luciferase and constructs used to abrogate or enhance MKP-1 expression (pSUPER.retro.MKP-1 or pFLAG-MKP1 WT, respectively). After incubation with or without 8Br–cAMP, cells were lysed and luciferase activity was measured. As shown in Fig. 4, shRNA against MKP-1 enhanced the effect of 8Br–cAMP on *Nr4a1* promoter

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

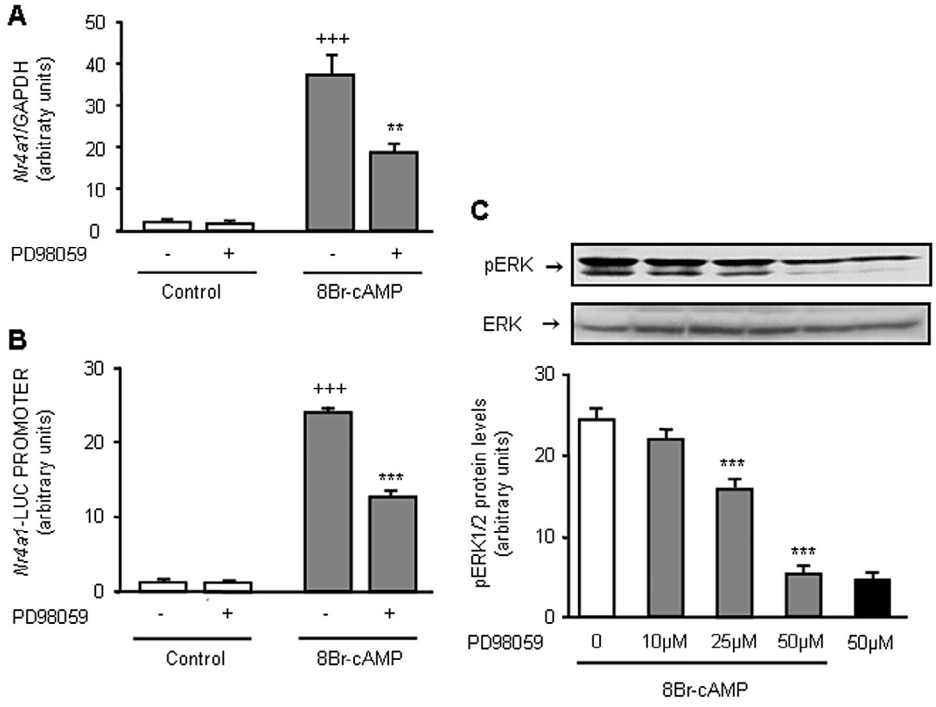

Fig. 2. Effect of ERK1/2 inhibition on *Nr4a1* expression in MA-10 Leydig cells. (A) Cells were serum-starved for 24 h, incubated with or without 50 μM PD98059 for 40 min and then stimulated or not with 0.5 mM 8Br–cAMP for 1 h. Total RNA was isolated and subjected to reverse transcription and real-time PCR using specific primers for *Nr4a1* and GAPDH as loading control. (B) Cells were transfected with a pGL-3 *Nr4a1* plasmid. They were serum-starved for 24 h, incubated with or without 50 μM PD98059 for 40 min, stimulated with 0.5 mM 8Br–cAMP for 4 h and finally lysed for luciferase activity evaluation. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments. $^{***}P < 0.001$, ** $P < 0.01$ vs. 8Br–cAMP-treated cells without PD98059, $^{+++}$ $P < 0.001$ vs. control without PD98059, by ANOVA followed by Tukey's test. (C) Cells were serum-starved for 24 h, incubated with 0, 10, 25 or 50 μM PD98059 for 40 min, stimulated or not with 0.5 mM 8Br–cAMP for 0.5 h and finally lysed for Western blot analyses of P-ERK1/2 levels using an anti-P-ERK1/2 antibody. Data are expressed in arbitrary units and represent the mean ± SEM of two independent experiments performed by duplicate. $^{***}P < 0.001$ vs. 8Br–cAMP-treated cells without PD98059.

activity, while MKP-1 over-expression produced the opposite effect (Fig. 4).

Preceding data suggest that variations in MKP-1 protein levels are translated into variations in cAMP-induced *Nr4a1* promoter activity. Therefore, post-translational modifications capable of modifying MKP-1 stability could have an impact on promoter regulation. Therefore, we first analyzed the effect of ERK-mediated phosphorylation on MKP-1 stability. To such end, pFLAG-MKP-1 construct encoding for chimera flag-MKP-1 WT was subjected to site-directed mutagenesis in order to obtain two DNA constructs mutated in the pairs of residues S359/S364 and S296/S323, encoding for flag-S359-S364-MKP-1 and flag-S296-S323-MKP-1, respectively. Cells transfected with these cDNA constructs were serum-starved for 24 h and then stimulated with 8Br–cAMP and treated according to a protocol for pulse-chase analysis used to evaluate the half life of MKP-1 and its mutated forms. During the first 30 min of chase time, flag-S296A-S323A-MKP-1 and flag-MKP-1 WT levels were similar, although the mutated form showed a longer half life compared to flag-MKP-1 WT (>120 min vs. 120 min) (Fig. 5A). In contrast, flag-S359A-S364A-MKP-1 showed a shorter half life (45 min) than WT chimera (Fig. 5B). It should be noted that, as soon as 30 min of chase time, flag-S359A-S364A-MKP-1 had decreased by 30% regarding control values, while flag-MKP-1 WT remained almost unchanged.

To test whether the expression of the different mutated forms of MKP-1 affects the stimulation of *Nr4a1* promoter by cAMP in a differential mode, luciferase activity was measured in samples from cells transfected with the empty vector (Mock) or with constructs for the expression of flag- MKP-1 WT, flag-S359A-S364A-MKP-1 or flag-S296-S323-MKP-1. In mock-transfected cells, 8Br–cAMP enhanced *Nr4a1* promoter activity nearly 20-fold, whereas the effect was lower in cells over-expressing MKP-1 (Fig. 6). In cells expressing flag-S296-S323-MKP-1, the stimulatory effect of cAMP was also smaller than in mock-transfected cells. Finally, the expression of flag-S359A-S364A-MKP-1 did not reduce the effect of 8Br–cAMP on *Nr4a1* promoter activity (Fig. 6), in agreement with the rapid decline observed in this mutant (Fig. 5B).

4. Discussion

Several studies have focused on the regulation of the orphan nuclear receptor Nur77, an important regulator of hormone-induced gene expression in steroidogenic cells. Crawford et al. have established a normal regulation of steroidogenesis in Nur77–/– mice (Crawford et al., 1995). However, it has been postulated that the Nur77–/– mice are rescued by compensatory expression of the other *Nr4a* subfamily members (Maxwell and Muscat, 2006). Here we used pharmacological and molecular tools to analyze the participation of ERK1/2 in cAMP-induced *Nr4a1* expression in MA-10 Leydig cells. Pharmacological evidence supports the notion that *Nr4a1* induction triggered by 8Br–cAMP is partially dependent on ERK1/2 activity. Consistent with this, changes in the levels of MKP-1 – which contribute to ERK1/2 dephosphorylation – result in changes in the magnitude of *Nr4a1* induction triggered by cAMP. Indeed, MKP-1 knockdown increases 8Br–cAMP-induced promoter activity, while its over-expression produces the opposite effect. Our findings also demonstrate that the stabilization of MKP-1 induced by cAMP in MA-10 Leydig cells involves several ERK phosphorylation consensus sites, and that post-translational modifications in these sites have an impact on the action of MKP-1 on *Nr4a1* expression.

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

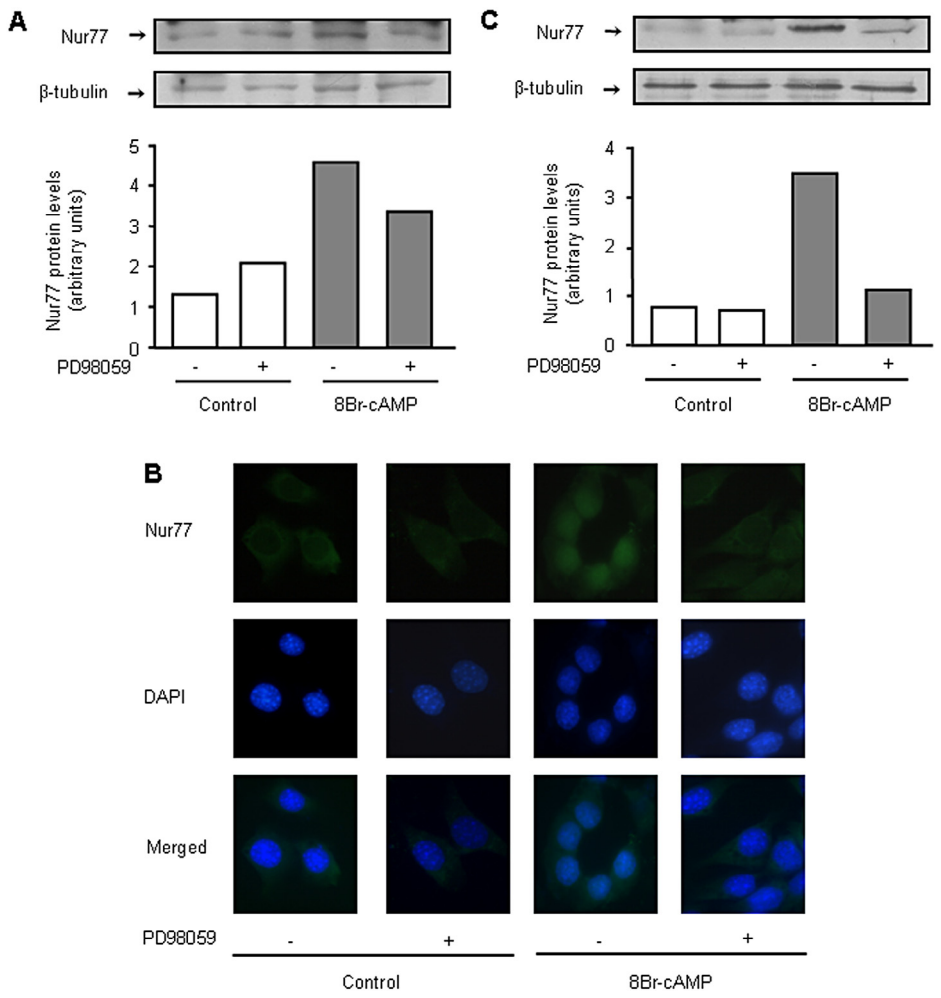

Fig. 3. Effect of AMPc and ERK1/2 inhibition on NUR77 protein expression. MA-10 Leydig cells (A) or Y1 adrenocortical cells (C) were serum-starved for 24 h, incubated with or without 50 μM PD98059 for 40 min and then stimulated or not with 0.5 mM 8Br–cAMP for 4 h. Then, cells were lysed and Nur77 protein levels were analyzed by Western blot. Membranes were stripped and β-tubulin was detected as loading control. Each panel shows representative immunoblots (upper panels). Integrated optical density of each specific band was quantitated and the values were normalized against β-tubulin abundance (lower panels). Data are expressed in arbitrary units. The figure shows a representative Western blot of two independent experiments. (B) Cells were grown on coverslips, serum-starved for 24 h and with or without 50 μM PD98059 for 40 min and then stimulated or not with 0.5 mM 8Br–cAMP for 4 h, fixed, stained with an antibody against Nur77 (green) and DAPI (blue) and then analyzed by fluorescence microscopy. The figure shows representative images of two separate experiments.

Earlier studies by our group demonstrated that LHR activation and cAMP rapidly lead to ERK1/2 phosphorylation and, subsequently, to MKP-1 and MKP-2 induction (Brion et al., 2011; Gomez et al., 2013). MKP-1, a product of an early gene, initiates ERK1/2 dephosphorylation; in contrast, MKP-2 is linked to the late stage of ERK1/2 dephosphorylation, in agreement with its slower induction kinetics. In view of this evidence, we have proposed that MKP-1 may modulate early ERK-dependent events involved in the regulation of steroidogenesis. In the same line, we have already demonstrated that MKP-1 reduces the effects of cAMP on ERK1/2 phosphorylation, StAR gene promoter activity, mRNA levels and steroidogenesis (Brion et al., 2011).

Because the target of MKP-1 in steroidogenesis remains elusive, we sought to establish whether ERK1/2 participates in the induction of *Nr4a1* by cAMP and, if so, whether this event could be modulated by MKP-1. Considering that the effect of cAMP on *Nr4a1* mRNA levels had already been described (Martin et al., 2009), we analyzed whether this messenger exhibited a similar temporal profile in cells cultured for 24 h in serum-deprived media. Indeed, in our system, messenger levels exhibited an increase of similar magnitude, even though the maximal effect elicited by 8Br–cAMP seemed to occur slightly earlier. Therefore, ERK1/2 participation

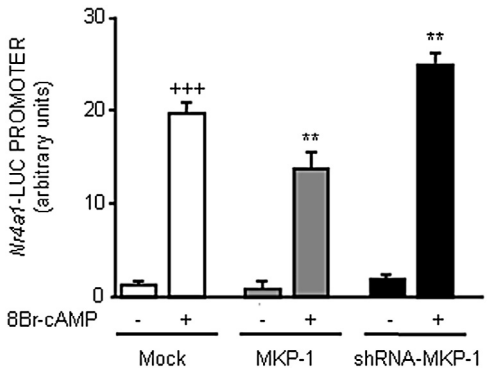

Fig. 4. Effect of MKP-1 on *Nr4a1* promoter activity in MA-10 Leydig cells. Cells were transfected with pFlag-MKP1 WT, shRNA-MKP-1 or the empty vector (Mock) with the pGL-3 *Nr4a1* vector and the control pRL *Renilla* luciferase vector. Cells were serum-starved for 24 h, then treated with 0.5 mM 8Br–cAMP for 4 h and finally lysed for luciferase activity evaluation. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments. *+++ P* < 0.001 vs. untreated-mock cells, ** *P* < 0.01 vs. mock cells stimulated with 8Br–cAMP, by ANOVA followed by Tukey's test.

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

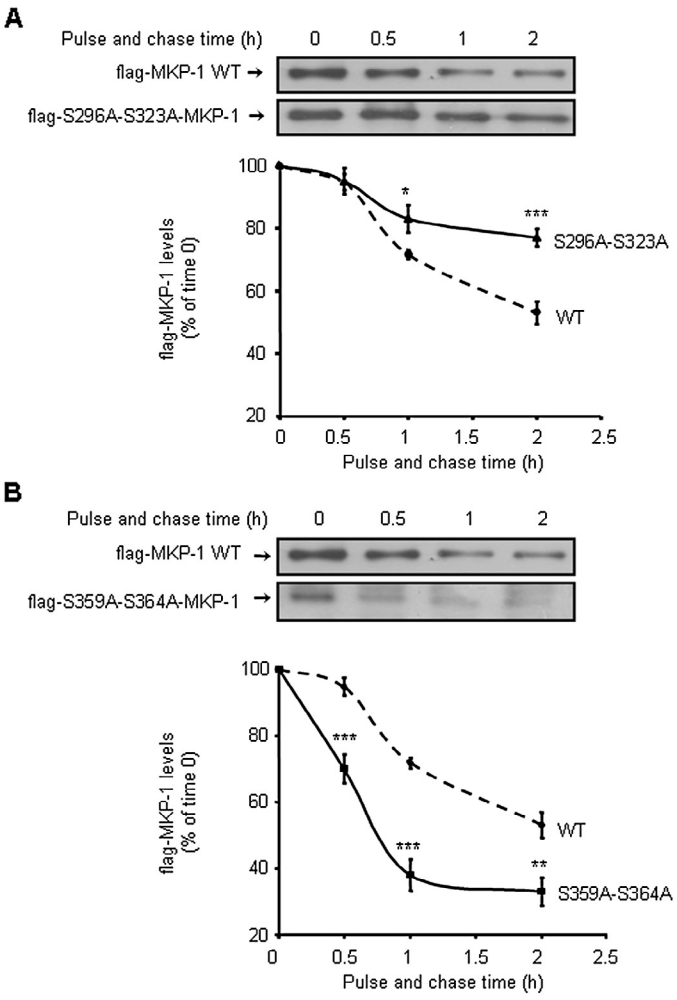

Fig. 5. Effect of ERK1/2 phosphorylation on MKP-1 stability in MA-10 Leydig cells. Cells were transfected with pFlag-MKP1 WT and pFlag-S296A-S323A-MKP-1 (A) or pFlag-S359A-S364A-MKP-1 (B) plasmids. They were serum-starved for 24 h, then incubated with 0.5 mM 8Br–cAMP for 30 minutes, later incubated with CHX (2 μg/ml) for different times (30 min of CHX = chase time 0) and finally lysed for Western blot analyses of flag-MKP-1 levels using an anti-flag antibody. Each panel shows a representative Western blot and the quantitative representation of the data of flag-MKP-1 levels normalized against β-tubulin signal. Data are expressed as the mean ± SEM of three independent experiments performed by duplicate. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ vs. WT, by ANOVA followed by Tukey's test.

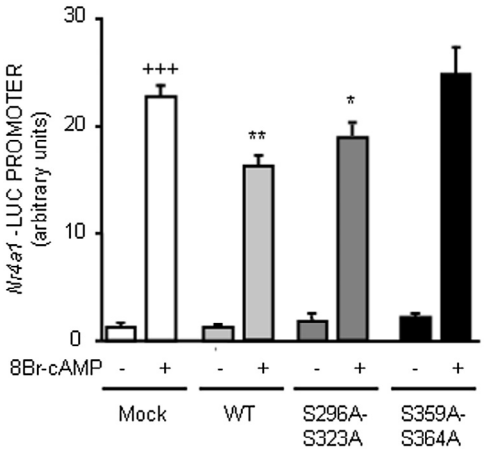

Fig. 6. Effect of ERK1/2-mediated MKP-1 phosphorylation on *Nr4a1* promoter activity in MA-10 Leydig cells. Cells were transfected with pFlag-MKP1 WT, pFlag-S359A-S364A-MKP-1, pFlag-S296A-S323A-MKP-1 or the empty vector (Mock) with the pGL-3 *Nr4a1* vector and the control pRL *Renilla* luciferase vector. They were serum-starved for 24 h, then treated with 0.5 mM 8Br–cAMP for 4 h and finally lysed for luciferase activity evaluation. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. mock cells stimulated with 8Br–cAMP, +++ $P < 0.001$ vs. untreated-mock cells, by ANOVA followed by Tukey's test.

in messenger induction was analyzed in cells stimulated with 8Br–cAMP for 1 h. Cell incubation with PD98059 reduced the stimulatory effect of 8Br–cAMP on messenger levels by 50%. Also, PD98059 produced an inhibitory effect of similar magnitude on the 8Br–cAMP-mediated increase in *Nr4a1* promoter activity and protein levels. In addition to these studies, we performed experiments based on the transient transfection of a DNA construct for the expression of flag-MKP-1. We have already demonstrated that this chimera is accumulated in 8Br–cAMP-treated cells and promotes ERK1/2 dephosphorylation (Brion et al., 2011). Again, results suggest that cAMP induces *Nr4a1* expression through a pathway that includes ERK1/2, which could be further supported by the fact that a shRNA against MKP-1 also significantly enhanced cAMP-mediated Nr4a1 promoter activation.

It has been reported that Nur77 is also inducible by ACTH and the second messenger analog dibutyryl cAMP in the ACTH-responsive adrenal cortical cell line Y1 (Davis and Lau, 1994). We thus tested the participation of ERK1/2 in 8Br–cAMP-induced Nur77 expression in Y1 adrenocortical cells. Our results show that 8Br–cAMP stimulation increased Nur77 levels and that MEK inhibition reduced this effect.

This work also extends the characterization of the post-translational regulation of MKP-1 by cAMP in MA-10 Leydig cells. Our results show that mutations in S359 and S364 reduce flag-MKP-1 half life, as we have previously proposed. This result suggests that ERK-mediated phosphorylation in these sites stabilizes MKP-1, in agreement with conclusions reached in several other systems (Brondello et al., 1999; Liu et al., 2009). A more novel result is the longer half life of flag-S296-S323-MKP-1 compared to the WT chimera, which supports the notion that protein phosphorylation in these sites leads to protein degradation. Therefore, our results suggest that the post-translational regulation of MKP-1 in MA-10 Leydig cells under hormonal stimulation implies a multi-site phosphorylation process. In addition, these results seem to impact *Nr4a1* regulation by cAMP. Indeed, flag-S359-S364-MKP-1, which is rapidly degraded, had no effect on this regulation, while WT chimera reduced the effect of 8Br–cAMP on *Nr4a1* promoter. In turn, flag-S296-S323-MKP-1 was more stable that WT chimera, although both produced an effect of similar magnitude. This is probably due to the fact that, even when these chimeras have different half lives, their levels could be undistinguishable during the critical period for *Nr4a1* induction, i.e. 30–60 min of stimulation.

Although our results prove ERK1/2 participation in *Nr4a1* expression by cAMP in MA-10 Leydig cells, the role of MAPK pathway in Nur77 expression in different systems is controversial and seems to be context- and stimulus-specific. Kovalovsky et al. (2002) demonstrated that PD98059 does not affect *Nr4a1* mRNA induction but blocks CRH- or cAMP-stimulated Nur transcriptional activity in corticotrophs. Moreover, these authors demonstrated that ERK1/2 stimulates phosphorylation and transactivation of Nur77 (Kovalovsky et al., 2002). On the other hand, it has been demonstrated that GnRH-induced transcriptional up-regulation of Nur77 in αT3-1 cells is dependent on calcium, protein kinase C and ERK signaling, while the transcriptional activity of Nur77 within gonadotropes in culture is regulated post-translationally by GnRH via PKC but not via ERK activity (Bliss et al., 2012). Also, both MEK and PKC activities have been described as modulators of *Nr4a1* mRNA levels induced by dopamine receptor agonists in the brain (Bourhis et al., 2008).

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

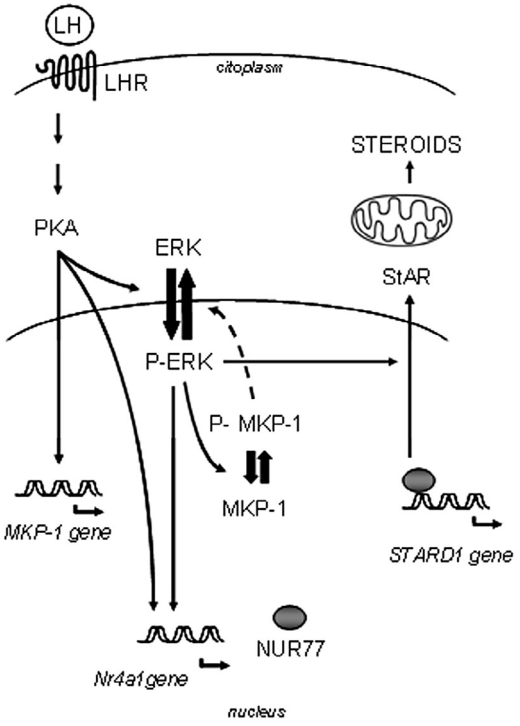

Fig. 7. Proposed model for the role of MKP-1 in steroidogenesis. LHR activation triggers PKA activation, which leads to ERK1/2 activation. PKA and ERK1/2 promote NUR77 expression and then StAR expression, which leads to an increase in steroid production. PKA also promotes an increase in MKP-1 protein levels by transcriptional activation and post-translational modifications which produce MKP-1 accumulation in the cell nucleus. In turn, MKP-1 protein dephosphorylates ERK1/2, generating a negative feedback on its activation and, consequently, on ERK-dependent events like NUR77 and StAR expression. Thus, MKP-1 induction participates in the "turn-off" of ERK1/2-dependent cellular events such as steroid production.

In MA-10 Leydig cells, Martin et al. have analyzed the kinases involved in the cAMP-mediated increase in Nur77 expression using several kinase inhibitors (Martin et al., 2009). These authors concluded that this process involves CAMKI (Ca^{2+}/calmodulin kinase I) but not ERK1/2 (Martin et al., 2008, 2009). However, it should be noted that these studies were performed with 10 μM PD98059, while our experiments were carried out with 50 μM PD98059, a concentration that blocks ERK1/2 phosphorylation in cAMP-stimulated MA-10 Leydig cells as well as other systems, e.g. ACTH-stimulated Y1 cells (Le and Schimmer, 2001).

Manna and Stocco have concluded that the role of MAPK signaling pathways in steroidogenesis is poorly understood, probably as a consequence of conflicting documents reporting stimulation, inhibition or no effect whatsoever (Manna and Stocco, 2011). We agree with these authors on the fact that the relationship between the regulation of MAPK-dependent StAR expression and steroid synthesis is complex and context-dependent. In this regard, a point that deserves attention is the different experimental conditions under which experiments are performed; for instance, the period of serum-starvation is a crucial variable to be considered.

In summary, we conclude that the induction of *Nr4a1* by cAMP in MA-10 Leydig cells is an ERK-dependent event. As such, it is not only up-regulated by ERK1/2 but also down-regulated by MKP-1. The effect of MKP-1 as a negative regulator of *Nr4a1* induction also highlights the post-translational regulation of this phosphatase by multi-site phosphorylation, whose activation by PKA-ERK cascade determines MKP-1 half life. Figure 7 depicts the regulation of *Nr4a1* by ERK1/2 and MKP-1.

Acknowledgements

We thank Dr. Mario Ascoli (University of Iowa College of Medicine, Iowa City, IA, USA) for kindly providing MA-10 cells, and Takashi Yazawa (Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Fukui, Japan) for generously providing pGL3-*Nr4a1* construct.

This work was supported in part by grants from Universidad de Buenos Aires (20020100100760 and 20020130100300) to CP and Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (PIP 112-200801-00209 and PIP 201101 00101) to CP. Cristina Paz holds research positions at CONICET.

References

Arakane, F., King, S.R., Du, Y., Kallen, C.B., Walsh, L.P., Watari, H., et al., 1997. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J. Biol. Chem. 272, 32656–32662.

Ascoli, M., 1981. Effects of hypocholesterolemia and chronic hormonal stimulation on sterol and steroid metabolism in a Leydig cell tumor. J. Lipid Res. 22, 1247–1253.

Bey, P., Gorostizaga, A.B., Maloberti, P.M., Lozano, R.C., Poderoso, C., Cornejo Maciel, F., et al., 2003. Adrenocorticotropin induces mitogen-activated protein kinase phosphatase 1 in Y1 mouse adrenocortical tumor cells. Endocrinology 144, 1399–1406.

Bliss, S.P., Navratil, A.M., Xie, J., Miller, A., Baccarini, M., Roberson, M.S., 2012. ERK signaling, but not c-Raf, is required for gonadotropin-releasing hormone (GnRH)-induced regulation of Nur77 in pituitary gonadotropes. Endocrinology 153, 700–711.

Bourhis, E., Maheux, J., Rouillard, C., Levesque, D., 2008. Extracellular signal-regulated kinases (ERK) and protein kinase C (PKC) activities are involved in the modulation of Nur77 and Nor-1 expression by dopaminergic drugs. J. Neurochem. 106, 875–888.

Boutros, T., Chevet, E., Metrakos, P., 2008. Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. Pharmacol. Rev. 60, 261–310.

Brion, L., Maloberti, P.M., Gomez, N.V., Poderoso, C., Gorostizaga, A.B., Mori Sequeiros Garcia, M.M., et al., 2011. MAPK phosphatase-1 (MKP-1) expression is up-regulated by hCG/cAMP and modulates steroidogenesis in MA-10 Leydig cells. Endocrinology 152, 2665–2677.

Brondello, J.M., Pouyssegur, J., McKenzie, F.R., 1999. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286, 2514–2517.

Cagnol, S., Chambard, J.C., 2010. ERK and cell death: mechanisms of ERK-induced cell death–apoptosis, autophagy and senescence. FEBS J. 277, 2–21.

Castilla, R., Gadaleta, M., Castillo, A.F., Duarte, A., Neuman, I., Paz, C., et al., 2008. New enzymes involved in the mechanism of action of epidermal growth factor in a clonal strain of Leydig tumor cells. Endocrinology 149, 3743–3752.

Caunt, C.J., Keyse, S.M., 2013. Dual-specificity MAP kinase phosphatases (MKPs): shaping the outcome of MAP kinase signalling. FEBS J. 280, 489–504.

Clark, B.J., Wells, J., King, S.R., Stocco, D.M., 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J. Biol. Chem. 269, 28314–28322.

Crawford, P.A., Sadovsky, Y., Woodson, K., Lee, S.L., Milbrandt, J., 1995. Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. Mol. Cell. Biol. 15, 4331–4336.

Davis, I.J., Lau, L.F., 1994. Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nurr-1 in the adrenal glands. Mol. Cell. Biol. 14, 3469–3483.

Gomez, N.V., Gorostizaga, A.B., Mori Sequeiros Garcia, M.M., Brion, L., Acquier, A., Gonzalez-Calvar, S.I., et al., 2013. MAPK phosphatase-2 (MKP-2) is induced by hCG and plays a role in the regulation of CYP11A1 expression in MA-10 Leydig cells. Endocrinology 154, 1488–1500.

Gorostizaga, A., Mori Sequeiros Garcia, M.M., Acquier, A., Gomez, N.V., Maloberti, P.M., Mendez, C.F., et al., 2013. Modulation of albumin-induced endoplasmic reticulum stress in renal proximal tubule cells by upregulation of mapk phosphatase-1. Chem. Biol. Interact. 206, 47–54.

Gyles, S.L., Burns, C.J., Whitehouse, B.J., Sugden, D., Marsh, P.J., Persaud, S.J., et al., 2001. ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. J. Biol. Chem. 276, 34888–34895.

Hirakawa, T., Ascoli, M., 2003. The lutropin/choriogonadotropin receptor-induced phosphorylation of the extracellular signal-regulated kinases in Leydig cells is mediated by a protein kinase a-dependent activation of ras. Mol. Endocrinol. 17, 2189–2200.

Inaoka, Y., Yazawa, T., Uesaka, M., Mizutani, T., Yamada, K., Miyamoto, K., 2008. Regulation of NGFI-B/Nur77 gene expression in the rat ovary and in Leydig tumor cells MA-10. Mol. Reprod. Dev. 75, 931–939.

Kovalovsky, D., Refojo, D., Liberman, A.C., Hochbaum, D., Pereda, M.P., Coso, O.A., et al., 2002. Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP:

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041

involvement of calcium, protein kinase A, and MAPK pathways. Mol. Endocrinol. 16, 1638–1651.

Le, T., Schimmer, B.P., 2001. The regulation of MAPKs in Y1 mouse adrenocortical tumor cells. Endocrinology 142, 4282–4287.

Lin, Y.W., Yang, J.L., 2006. Cooperation of ERK and SCFSkp2 for MKP-1 destruction provides a positive feedback regulation of proliferating signaling. J. Biol. Chem. 281, 915–926.

Liu, R., Zheng, H.Q., Zhou, Z., Dong, J.T., Chen, C., 2009. KLF5 promotes breast cell survival partially through fibroblast growth factor-binding protein 1-pERK-mediated dual specificity MKP-1 protein phosphorylation and stabilization. J. Biol. Chem. 284, 16791–16798.

Manna, P.R., Stocco, D.M., 2011. The role of specific mitogen-activated protein kinase signaling cascades in the regulation of steroidogenesis. J. Signal Transduct. 2011, 821615.

Martin, L.J., Boucher, N., Brousseau, C., Tremblay, J.J., 2008. The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca2+/calmodulin-dependent protein kinase I. Mol. Endocrinol. 22, 2021–2037.

Martin, L.J., Boucher, N., El-Asmar, B., Tremblay, J.J., 2009. cAMP-induced expression of the orphan nuclear receptor Nur77 in MA-10 Leydig cells involves a CaMKI pathway. J. Androl. 30, 134–145.

Maxwell, M.A., Muscat, G.E., 2006. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. Nucl. Recept. Signal. 4, e002.

Poderoso, C., Converso, D.P., Maloberti, P., Duarte, A., Neuman, I., Galli, S., et al., 2008. A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis. PLoS ONE 3, e1443.

Podesta, E.J., Dufau, M.L., Catt, K.J., 1976. Adenosine 3′,5′-monophosphate-dependent protein kinase of Leydig cells: in vitro activation and relationship to gonadotropin action upon cyclic AMP and steroidogenesis. FEBS Lett. 70, 212–216.

Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. Oncogene 26, 3100–3112.

Stocco, C.O., Lau, L.F., Gibori, G., 2002. A calcium/calmodulin-dependent activation of ERK1/2 mediates JunD phosphorylation and induction of nur77 and 20alpha-hsd genes by prostaglandin F2alpha in ovarian cells. J. Biol. Chem. 277, 3293–3302.

Stocco, D.M., Clark, B.J., 1996. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. Biochem. Pharmacol. 51, 197–205.

Tong, X.K., Hamel, E., 2007. Transforming growth factor-beta 1 impairs endothelin-1-mediated contraction of brain vessels by inducing mitogen-activated protein (MAP) kinase phosphatase-1 and inhibiting p38 MAP kinase. Mol. Pharmacol. 72, 1476–1483.

Please cite this article in press as: Mercedes Mori Sequeiros Garcia, Alejandra Gorostizaga, Laura Brion, Silvia I. González-Calvar, Cristina Paz, cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.01.041