Calcium-dependent *Nr4a1* expression in mouse Leydig cells requires distinct AP1/CRE and MEF2 elements

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Abstract

The nuclear receptor NR4A1 is expressed in steroidogenic Leydig cells where it plays pivotal roles by regulating the expression of several genes involved in steroidogenesis and male sex differentiation including *Star*, *HSD3B2*, and *InsI3*. Activation of the cAMP and Ca²⁺ signaling pathways in response to LH stimulation leads to a rapid and robust activation of *Nr4a1* gene expression that requires the Ca²⁺/CAMK1 pathway. However, the downstream transcription factor(s) have yet to be characterized. To identify potential Ca²⁺/CaM effectors responsible for hormone-induced *Nr4a1* expression, MA-10 Leydig cells were treated with forskolin to increase endogenous cAMP levels, dantrolene to inhibit endoplasmic reticulum Ca²⁺ release, and W7 to inhibit CaM activity. We identified Ca²⁺-responsive elements located in the discrete regions of the *Nr4a1* promoter, which contain binding sites for several transcription factors such as AP1, CREB, and MEF2. We found that one of the three AP1/CRE sites located at –255 bp is the most responsive to the Ca²⁺ signaling pathway as are the two MEF2 binding sites at –315 and –285 bp. Furthermore, we found that the hormone-induced recruitment of phospho-CREB and of the co-activator p300 to the *Nr4a1* promoter requires the Ca²⁺ pathway. Lastly, siRNA-mediated knockdown of CREB impaired NR4A1 expression and steroidogenesis. Together, our data indicate that the Ca²⁺ signaling pathway increases *Nr4a1* expression in MA-10 Leydig cells, at least in part, by enhancing the recruitment of coactivator most likely through the MEF2, AP1, and CREB transcription factors thus demonstrating an important interplay between the Ca²⁺ and cAMP pathways in regulating *Nr4a1* expression.

Key Words

- ▶ steroidogenesis
- ▶ Ca²⁺
- ▶ NR4A1
- ▶ AP1
- ▶ CREB
- ▶ MEF2
- ▶ p300
- ▶ Leydig cells

*Journal of Molecular
Endocrinology*
(2016) **56**, 151–161

Introduction

Binding of LH to its receptor on the surface of Leydig cells triggers the production of several second messengers including cAMP, Ca²⁺, DAG, and IP₃, which in turn activate various transcription factors leading to increased gene expression and ultimately a physiological response, i.e. enhanced steroid hormone biosynthesis. Previously, the classical cAMP pathway

was thought to account for most of the LH-induced steroidogenesis in Leydig cells (reviewed in [Hansson et al. 2000](#)). However, several reports have also established the requirement of a transient increase in cytoplasmic Ca²⁺ following trophic hormone stimulation for proper steroidogenesis to occur ([Dufau et al. 1984](#), [Sullivan & Cooke 1986](#)).

Two L-type Ca²⁺ channels receptors are present in the endoplasmic reticulum (ER) of murine Leydig cells: the ryanodine receptors (RyRs) I, II, and III, and the inositol triphosphate receptors (IP3Rs) I, II, and III (Costa *et al.* 2010). Activation of the RyRs has been shown to be the primary mediator of the Ca²⁺ signaling pathway in Leydig cells steroidogenesis (Costa *et al.* 2010, Abdou *et al.* 2013). We recently have shown that the RyR and Ca²⁺-calmodulin (CaM) inhibitors, ryanodine and W7, respectively, impair steroidogenesis in the MA-10 Leydig cell line through a decrease in the expression and promoter activity of STAR (Abdou *et al.* 2013). The main Ca²⁺/CaM kinase involved in *Star* expression in Leydig cells is CAMKI (Martin *et al.* 2008). Furthermore, the expression and promoter activity of *Nr4a1*, a mediator of hormone-stimulated *Star* expression (Martin *et al.* 2008, Abdou *et al.* 2013), were also compromised upon disruption of the Ca²⁺ pathway (Martin *et al.* 2008, Abdou *et al.* 2013). Thus, our data identified a transcriptional cascade initiated by Ca²⁺ release from ER stores in the regulation of steroid hormone biosynthesis in Leydig cells.

The NR4A1 (NUR77, NGFI-B) nuclear receptor is part of the subfamily of NR4A nuclear receptors along with NR4A2 (NURR1) and NR4A3 (NOR1). Their expression is modulated by several agents, including but not limited to trophic hormones, Ca²⁺, and growth factors (reviewed in (Li *et al.* 2006)). NR4A1 is expressed in adrenal and gonadal cells, where it regulates the expression of several genes involved in steroidogenesis including *Star* (Martin *et al.* 2008), *Cyp17a1* (Zhang & Mellon 1997), *HSD3B2* (Bassett *et al.* 2004a, Martin & Tremblay 2005), and *Cyp11b2* (Bassett *et al.* 2004b).

In steroidogenic Leydig cells, NR4A1 protein and *Nr4a1* mRNA levels are induced as early as 30 min post LH/hCG stimulation and peak at 2 h before rapidly decreasing to undetectable levels by 3 h (Song *et al.* 2001). A similar kinetic was observed in MA-10 Leydig cells treated with forskolin (Fsk) (Martin *et al.* 2008). LH/Fsk-induced *Nr4a1* expression is blunted in the presence of inhibitors of RyRs and CaM (Abdou *et al.* 2013), protein kinase A (PKA) (Song *et al.* 2001, Martin *et al.* 2009), phosphoinositide-3 kinase (PI3K) (Song *et al.* 2001), and CAMK (Martin *et al.* 2009).

While it is known that the Ca²⁺/CAMKI signaling pathways is important for *Nr4a1* expression in Leydig cells (Martin *et al.* 2008, Abdou *et al.* 2013), the specific transcription factors downstream of this pathway, and thus affected by the inhibitors ryanodine and W7, remain poorly characterized. In this study, we found that several response elements, including AP1, CRE,

and MEF2, within the proximal *Nr4a1* promoter are sensitive to the disruption of the Ca²⁺ signaling pathway by either dantrolene, which inhibits intracellular Ca²⁺ release by the ER, and W7, a CaM inhibitor. Mutation of these elements abolished the effect of either inhibitors. We also found that the Fsk-induced recruitment of phosphorylated CREB and of the ubiquitous coactivator p300 to the proximal *Nr4a1* promoter was decreased following treatment with either dantrolene or W7. Together, these results suggest that the transcription factors that bind to these elements activate *Nr4a1* gene expression in MA-10 Leydig cells in a Ca²⁺-dependent manner by modulating the recruitment of the coactivator p300.

Materials and methods

Cell culture

The mouse MA-10 Leydig cell line (Ascoli 1981) was provided by Dr Mario Ascoli (University of Iowa, Iowa City, Iowa) and maintained in DMEM-F12 supplemented with penicillin and streptomycin, 15% horse serum, and incubated at 37°C in 5% CO₂.

Chemicals

The RyR inhibitor dantrolene and the calmodulin (CaM) inhibitor W7 hydroxychloride were obtained from Tocris Bioscience (Minneapolis, MN) and previously validated in MA-10 Leydig cells (Abdou *et al.* 2013). Forskolin (Fsk) and 8-bromo-cAMP (8Br-cAMP) were purchased from Sigma-Aldrich.

Plasmids

The -331 to +50 bp rat *Nr4a1* promoter and the various NIR-A, NIR-B, and NIR-C reporter constructs were described previously (Martin *et al.* 2009). The three TGCGTCA AP1/CRE elements located at -255, -233, and -91 bp were mutated to TGCacCA by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) along with the following three pairs of oligonucleotides (the AP1/CRE site is underlined and the mutated nucleotides are in lowercase): -255 bp sense 5'-ACA ATC CGC GCT CCC TGC acC AAT GGA ACC CCG CGT GCG-3' and -255 antisense 5'-CGC ACG CGG GGT TCC ATT Ggt GCA GGG AGC GCG GAT TGT-3'; -233 bp sense 5'-CAA TGG AAC CCC GCG TGC acC ACG CGC GCA GAC ATT CC-3'

and –233 bp antisense 5'-GGA ATG TCT GCG CGC GTG gtG CAC GCG GGG TTC CAT TG-3'; –91 bp sense 5'-GCT CGC CGG GCC GTG TGC acC AGT GGC GCC CCC GCC CCT C-3' and –91 bp antisense 5'-GAG GGG CGG GGG CGC CAC TGg tGC ACA CGG CCC GGC GAG C-3'. The constructs harboring mutations in the two MEF2 elements located at –319 and –285 bp were described previously (Daems *et al.* 2014). The expression vector for MEF2D was described previously (Daems *et al.* 2014). The cJUN expression vector (Teyssier *et al.* 2001) was obtained from Dr Dany Chabos (Institut National de la Santé et de la Recherche Médicale, Endocrinologie Moléculaire et Cellulaire des Cancers, Montpellier, France). The CREB expression vector (Mayr & Montminy 2001) was provided by Dr. Marc Montminy (The Salk Institute for Biological Studies, La Jolla, California).

Protein purification and western blots

Nuclear extracts and western blots were performed as described previously (Martin *et al.* 2008, Abdou *et al.* 2014). For each experiment, MA-10 Leydig cells were cultured in serum-free media in the presence of either vehicle, Fsk (10 µM) alone, Fsk + dantrolene (150 µM), Fsk + W7 (40 µM) for 1 h before nuclear extracts preparation. Each experiment was performed at least 3 times. The antisera used for detection of NR4A1 (M-210), LAMIN B (C-20) and CREB (H-74) were purchased from Santa Cruz Biotechnology.

Transfections and reporter assays

Mouse MA-10 Leydig cells were transfected, lysed, and lysates analyzed as described previously (Martin *et al.* 2008, Martin *et al.* 2009). The cells were treated with either vehicle, 8Br-cAMP (0.5 mM), 8Br-cAMP + dantrolene (150 µM), or 8Br-cAMP + W7 (40 µM) for 4 h before being lysed. When indicated, 100 ng of expression vector (cJUN, CREB, and MEF2D) was also co-transfected with the luciferase reporter construct.

Chromatin immunoprecipitation assay

MA-10 Leydig cell were treated for 30 min with vehicle, Fsk (10 µM), Fsk + dantrolene (150 µM), or Fsk + W7 (40 µM) in serum-free media. The cells were next cross-linked with 1% formaldehyde for 10 min at 37 °C, washed twice with cold PBS and then scraped and transferred into microtubes. The experimental procedures can be found in Abdou *et al.* (2014). Five µg of either IgG (negative

control) or anti-p300, anti-CREB, or anti-pCREB antisera (Santa Cruz Biotechnology) were used as described previously (Abdou *et al.* 2014). PCR amplification of the –284 to –3 bp region of murine *Nr4a1* promoter was used to confirm the recruitment of the factors.

Transfections of siRNAs

For knockdown experiments, 150 nM of siRNA oligos were used per 60 mM plate along with 16 µL of Jetprime/200 µL Jetprime buffer. In some cases, the amounts were scaled down proportionally when smaller wells were used. Knockdown of CREB was achieved by using a combination (1:1:1 ratio) of the following three pairs of siRNA oligos

Figure 1

Inhibition of the Ca²⁺-sensing protein calmodulin (CaM) and the ryanodine receptors impair hormone-induced NR4A1 expression in MA-10 Leydig cells. (A) NR4A1 protein levels were determined by western blot using nuclear extracts from MA-10 Leydig cells treated for 1 h with either vehicle, Fsk (10 µM), Fsk + dantrolene (150 µM), or Fsk + W7 (40 µM). Representative blots from three independent experiments are shown. LAMIN B was used as a loading control. The NR4A1 protein is seen as two bands where one represents a phosphorylated form, as previously shown (Fahrner *et al.* 1990, Martin *et al.* 2008). (B) MA-10 Leydig cells were transfected with a –331 bp *Nr4a1* reporter and treated with vehicle, 8Br-cAMP (0.5 mM), 8Br-cAMP + dantrolene (150 µM), or 8Br-cAMP + W7 (40 µM) as indicated. Results are shown as fold activation over vehicle ± S.E.M. Different letters indicate statistically significant differences ($P < 0.05$).

(only the sense strand is shown): 1st GCC CAG CAA CCA AGU UGU UGU UCA A, 2nd AGG GAG GAG CAA UAC AGC UGG CUA A, 3rd CAC GGA AGA GAG AGG UCC GUC UAA U.

Progesterone quantification

Progesterone levels from MA-10 Leydig cells were quantified by ELISA (Cayman Chemical) as described previously (Abdou *et al.* 2014). For basal progesterone levels, 50 μ L of media was diluted 41 times, 50 μ L of which was used in the assay. For Fsk-stimulated progesterone levels, 50 μ L of the media was diluted 441 times and 50 μ L of the dilution was subsequently used. Values were normalized to the proteins levels extracted from each well and results presented as ng/mg of total proteins.

Statistical analysis

For the multiple group comparisons, statistical analyses were performed by using a one-way ANOVA followed by the Bonferroni *post hoc* test. For all single comparisons between two experimental groups, one-tailed paired Student's *t* tests were performed. A *P* < 0.05 was considered to be significant. All statistical analyses were

done using SigmaStat software package (Systat Software Inc., San Jose, CA).

Results

Blocking Ca²⁺ release from intracellular stores inhibits Fsk-induced *Nr4a1* expression and promoter activity

In a previous study, we showed that RyR inhibition using Ryanodine, which inhibits Ca²⁺ release from internal stores, blunts hormone-induced steroidogenesis and *Star* gene expression, and that NR4A1 expression was also decreased (Abdou *et al.* 2013). To gain more insights into the mechanisms involved in Ca²⁺-dependent NR4A1 expression in response to forskolin (Fsk)/cAMP, we evaluated the effect of dantrolene and W7, which are known inhibitors of Ca²⁺ release and CaM activity, respectively. The effects of the inhibitors were first assessed on NR4A1 protein levels. As expected, a strong increase in NR4A1 expression was observed in the presence of Fsk (Fig. 1A). However, this increase was significantly reduced in the presence of either dantrolene or W7 (Fig. 1A). Furthermore, both dantrolene and W7 decreased the cAMP-induced activity of the -331 bp *Nr4a1* reporter by ~50% and

Figure 2

The Ca²⁺ pathway targets several regions in the *Nr4a1* promoter. MA-10 Leydig cells were transiently transfected with various *Nr4a1* reporter constructs: -331 to +50 bp (NIR-ABC), -331 to -233 (NIR-A), -233 to -122 (NIR-B), -122 to +50 (NIR-C), and -65 to +50 (minimal promoter). Cells were then treated with either vehicle, 8Br-cAMP (0.5 mM), 8Br-cAMP + dantrolene (150 μ M, left panel), or 8Br-cAMP + W7 (40 μ M, right panel). Results are shown as fold activation over vehicle $\pm$ S.E.M. Different letters indicate statistically significant differences (*P* < 0.05).

~80%, respectively (Fig. 1B). This is in agreement with the previously published data using a -747 to +50 bp *Nr4a1* reporter (Abdou *et al.* 2013). Collectively, these data indicate that hormone/Fsk/cAMP-induced NR4A1 expression requires adequate Ca²⁺ signaling in the MA-10 Leydig cell line.

It was previously shown that the *Nr4a1* proximal promoter (-331 to +50 bp) can be divided into three regions with distinct activities: NIR-A (-331 to -233 bp), NIR-B (-233 to -121 bp), and NIR-C (-121 to -65 bp) (Martin *et al.* 2009). The NIR-A and NIR-C regions are hormone/cAMP responsive, while NIR-B mediates most of the basal promoter activity (Martin *et al.* 2009). However, the Ca²⁺-responsive region(s) have never been studied. To better locate the region within the *Nr4a1* promoter that is targeted by the Ca²⁺ signaling pathway, the inhibitors dantrolene and W7 were used with the NIR reporter constructs. As shown in Fig. 2, the cAMP-induced activity of the NIR-A and NIR-C regions (the cAMP-responsive regions) were the most affected by dantrolene

and W7 when compared with NIR-B region or to the minimal promoter which are essentially unresponsive to cAMP (Fig. 2). These results indicate that the Ca²⁺-responsive elements are mainly located in the NIR-A and NIR-C regions of the *Nr4a1* promoter.

Ca²⁺ responsiveness of the *Nr4a1* promoter requires intact AP1/CRE and MEF2 elements

In silico analysis of these regions revealed the presence of binding sites for several transcription factors. These include AP1/CRE half sites located at -255 bp (NIR-A), -233 bp (NIR-B), and -91 bp (NIR-C), as well as MEF2 elements located at -319 bp and -285 bp (NIR-A). The AP1 and CREB transcription factors have been shown to bind and activate the *Nr4a1* promoter mostly through these AP1/CRE (Inaoka *et al.* 2008), while MEF2 is recruited to activate the *Nr4a1* promoter via the two MEF2 elements (Daems *et al.* 2014). Importantly, these transcription factors (AP1, CREB, and MEF2) are known to be activated by the Ca²⁺ pathway in other systems (Tsuda *et al.* 1986,

Figure 3

The Ca²⁺ signaling pathway acts primarily via the -255 bp AP1/CRE element. MA-10 Leydig cells were transiently transfected with the rat NIR-ABC *Nr4a1* promoter region either wild type or harboring mutations (individual or combined as indicated by a large X) in the three AP1/CRE elements (-255 bp, -233 bp, -91 bp). Cells were then treated with either vehicle, 8Br-cAMP (0.5 mM), 8Br-cAMP + dantrolene (150 μM, left panel), or 8Br-cAMP + W7 (40 μM, right panel). Results are shown as fold activation over vehicle ± s.e.m. Different letters indicate statistically significant differences ($P < 0.05$).

Sheng *et al.* 1990, Sheng *et al.* 1991, Youn & Liu 2000, Stocco *et al.* 2002, Linseman *et al.* 2003).

We therefore tested whether the Ca²⁺ responsiveness of the *Nr4a1* promoter involves the AP1/CRE and MEF2 elements. When the AP1/CRE element at –255 bp was mutated in the context of the –331 bp *Nr4a1* reporter, the cAMP-dependent stimulation was significantly reduced from 6- to 9-fold to 2-fold (Fig. 3). Consistent with this, inhibition of either Ca²⁺ release from the ER by dantrolene or of CaM activity by W7 had no effect on the activity of –331 bp *Nr4a1* promoter mutated in the –255 AP1/CRE element (Fig. 3). Mutation of the other AP1/CRE sites at –233 bp and –91 bp did not affect cAMP responsiveness of the promoter, and was still repressed by dantrolene and W7 as efficiently as the WT reporter (Fig. 3). However, simultaneous mutation of both –233 bp and –91 bp AP1/CRE sites showed reduced cAMP responsiveness (3 vs 6- to 9-fold for WT) and a reduced sensitivity to the inhibitors dantrolene and W7 (25% reduction compared with 50–75% for the WT reporter; $P < 0.05$ one-tailed paired Student's *t* test) (Fig. 3). Lastly, a –331 bp *Nr4a1* reporter harboring mutations in all three AP1/CRE sites was no longer responsive to cAMP and insensitive to dantrolene and W7 (Fig. 3). Together, the results suggest that of the three AP1/CRE sites, the one located at –255 bp is the most responsive to the Ca²⁺-signaling pathway in MA-10 Leydig cells.

Several reports identified MEF2 as an essential effector of the Ca²⁺-signaling pathway in various cell types including cardiomyocytes and myoblasts (Lu *et al.* 2000, McKinsey *et al.* 2000, Passier *et al.* 2000), T-cells (Woronicz *et al.* 1995), and more recently steroidogenic Leydig cells (Daems *et al.* 2014). To test whether the Ca²⁺-signaling pathway regulates *Nr4a1*

promoter activity through MEF2 transcription factors, we treated the MA-10 Leydig cells with cAMP and with either dantrolene or W7 following the transfection of a –331 bp *Nr4a1* reporter, either wild-type or mutated, in both MEF2 elements. As shown in Fig. 4, *Nr4a1* promoter activity was increased in the presence of cAMP and this cAMP-mediated increase was reduced (from 6- to 4-fold) when the MEF2 elements were mutated, as expected (Daems *et al.* 2014). Consistent with data presented in Figs 2 and 3, the inhibitors dantrolene and W7 repressed the cAMP-induced activity of the wild type –331 bp *Nr4a1* reporter. However, dantrolene and W7 had no effect on the cAMP responsiveness of the –331 bp reporter mutated in the MEF2 elements (Fig. 4). The fact that the remaining cAMP responsiveness of the MEF2-mutated –331 bp reporter was unaltered by the inhibitors indicates that the Ca²⁺ pathway also acts via the MEF2 elements in the *Nr4a1* promoter.

We next tested whether the Ca²⁺ signaling pathway acts, at least in part, via the transcription factors cJUN, CREB, and MEF2D, which are known to bind to the AP1/CRE and MEF2 elements in the *Nr4a1* promoter in MA-10 steroidogenic cells (Inaoka *et al.* 2008, Martin *et al.* 2009, Daems *et al.* 2014). This was assessed by overexpressing either cJUN, CREB, or MEF2D in order to determine if this could rescue the repressive effects of dantrolene and W7 on the cAMP-induced *Nr4a1* promoter activity. As shown in Fig. 5, the repressive effects of dantrolene and W7 were lost in the presence of exogenously expressed cJUN, CREB, or MEF2D. This indicates that these transcription factors are likely downstream effectors of the Ca²⁺ pathway in MA-10 Leydig cells.

Figure 4

The Ca²⁺ signaling pathway requires the MEF2 elements within the proximal *Nr4a1* promoter. MA-10 Leydig cells were transiently transfected with a rat NIR-ABC *Nr4a1* promoter region either wild type or harboring mutations (depicted by a large X) in the two MEF2 elements (–319 bp and –285 bp) and treated with either vehicle (open bars), 8Br-cAMP (0.5 mM, gray bars), 8Br-cAMP + dantrolene (150 μM, black bars), or 8Br-cAMP + W7 (40 μM, hatched bars). Results are shown as fold activation over vehicle ± s.e.m. Different letters indicate statistically significant differences ($P < 0.05$).

The Ca²⁺-signaling pathway modulates phospho-CREB and p300 recruitment to the *Nr4a1* promoter

In other cells, the MEF2 transcription factors regulates *Nr4a1* expression by modulating the recruitment of transcriptional co-activators such as p300 (Youn *et al.* 2000) and co-repressors including histone deacetylases (HDACs) 1, 2, 4, and 5 (Lu *et al.* 2000, McKinsey *et al.* 2000, Youn & Liu 2000). The leucine-zipper factors cJUN and CREB, known to dimerize (Hai & Curran 1991), activate transcription in a CBP/p300-dependent manner in other cells ((Kim *et al.* 2005, Yamasaki *et al.* 2009) and reviewed in (Vo & Goodman 2001)). As these transcription factors are expressed in Leydig cells, regulate *Nr4a1* expression, and their binding sites are targeted by the Ca²⁺ pathway, we used chromatin immunoprecipitation (ChIP) assays to test the possibility that the Ca²⁺ pathway might modulate p300 and CREB recruitment to the proximal *Nr4a1* promoter (Fig. 6A). As shown in Fig. 6B, Fsk treatment led to an increase in p300

recruitment to the *Nr4a1* promoter as previously observed in MA-10 Leydig cells (Abdou *et al.* 2014). However, this increase was abolished in the presence of dantrolene or W7 (Fig. 6B). While CREB recruitment to the *Nr4a1* promoter was unchanged following Fsk stimulation, phosphorylated CREB (pCREB) recruitment was increased (Fig. 6C). This increase in pCREB recruitment was, however, lost in the presence of dantrolene or W7 (Fig. 6C). This indicates that the Ca²⁺-signaling pathway increases *Nr4a1* gene

Figure 5

Overexpression of cJUN, CREB, or MEF2D rescues the inhibitory effects of dantrolene and W7 on the proximal *Nr4a1* promoter. MA-10 Leydig cells were transiently transfected with the rat NIR-ABC *Nr4a1* promoter region along with an empty expression vector or expression vectors for cJUN, CREB, or MEF2D as indicated and treated with either 8Br-cAMP (0.5 mM, gray bars), 8Br-cAMP + dantrolene (150 μM, black bars), or 8Br-cAMP + W7 (40 μM, hatched bars). Results are shown as % activity ± S.E.M. relative to the activity of the reporter treated with 8Br-cAMP, which was arbitrarily set at 100%. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 6

Recruitment of p300 and pCREB to the proximal *Nr4a1* promoter in MA-10 Leydig cells requires an intact Ca²⁺ signaling pathway. (A) Schematic representation of the *Nr4a1* locus that was targeted in the ChIP assays. The position of the AP1/CRE (gray rectangles) and MEF2 (black rectangles) elements is shown. The 281 bp region that was amplified is represented by the dotted line. (B) MA-10 Leydig cells were treated for 30 min with DMSO, Fsk (10 μM), Fsk + dantrolene (150 μM), or Fsk + W7 (40 μM) in a serum-free media and chromatin immunoprecipitation (ChIP) experiments were performed to assess p300 recruitment to the -284 to -3 bp region of the *Nr4a1* promoter. (C) The conditions described in (B) were used to assess CREB and phosphorylated CREB (pCREB) recruitment to the -284 to -3 bp region of the *Nr4a1* promoter. A non-specific antibody (IgG) was used as a negative control and 1% of the sonicated extract was used as input.

expression, at least in part, by enhancing the recruitment of co-activator most likely through the AP1 (cJUN) and pCREB transcription factors.

Depletion of CREB in MA-10 Leydig cells impairs NR4A1 expression and steroidogenesis

As the knockdown of MEF2A/2D has already been shown to reduce *Nr4a1* expression and steroid hormone production in MA-10 Leydig cells (Daems *et al.* 2014, Daems *et al.* 2015), the contribution of the cJUN/CREB complex to these processes was next evaluated. Activated cJUN and CREB have been correlated with maximal steroidogenesis and activation of *Nr4a1* promoter activity (Martin & Tremblay 2009, Manna *et al.* 2011). As CREB and cJUN dimerize and bind to target promoters (Hai & Curran 1991), we assessed

the impact of CREB knockdown on NR4A1 expression and steroidogenesis. As shown in Fig. 7A, siRNA-mediated knockdown of CREB was sufficient to significantly reduce the Fsk/cAMP-mediated increase in NR4A1 protein levels in MA-10 Leydig cells. Depletion of CREB also decreased Fsk-induced progesterone production (Fig. 7B). These results indicate that the pro-steroidogenic role of CREB occurs primarily in cAMP/Ca²⁺-stimulated cells and position CREB upstream of NR4A1 in a regulatory cascade in hormone-induced MA-10 Leydig cells.

Discussion

While studies with cells derived from rodents (Sullivan & Cooke 1986, Kumar *et al.* 1994, Lee *et al.* 2011), bovine (Cherradi *et al.* 1997), and humans (Clark *et al.* 1995) have revealed that the Ca²⁺ signaling pathway is crucial to steroid hormone synthesis, health, and reproductive function, very little is known about the transcriptional cascade initiated upon increase in cytoplasmic Ca²⁺ levels. We and others have recently showed that the Ca²⁺-dependent increase in steroid synthesis requires the acute expression of STAR in Leydig cells (Manna *et al.* 1999, Abdou *et al.* 2013). This explains, at least in part, the mechanism of actions of several antihypertensive drugs targeting Ca²⁺ channels that are known to impair steroidogenesis and spermatogenesis in rodents (Almeida *et al.* 2000, Lee *et al.* 2011). Surprisingly, a detailed characterization of the Ca²⁺-mediated *Star*-dependent acute steroidogenesis is still lacking. Our previous study was the first to identify NR4A1, but not NR5A1, as one of the main downstream effector of Ca²⁺-dependent increase in *Star* expression and thus steroidogenesis in Leydig cells (Abdou *et al.* 2013). In this study, we identified key downstream effectors of the Ca²⁺-signaling pathway involved in the transcriptional regulation of *Nr4a1* by mapping Ca²⁺-responsive elements located at -319 (MEF2), -285 (MEF2), and -255 (CRE/AP1) of the rat *Nr4a1* gene. Lastly, we identified p300 and pCREB as regulators targeted by the Ca²⁺ pathway to stimulate *Nr4a1* gene expression in MA-10 Leydig cells, and showed that CREB is required for NR4A1 expression and steroidogenesis.

Figure 7

siRNA-mediated knockdown of CREB impairs NR4A1 expression and steroidogenesis in MA-10 Leydig cells. (A) CREB and NR4A1 protein levels were determined by western blot using nuclear extract from MA-10 Leydig cells 2 days post-transfection of either control siRNAs (Ctrl) or siRNAs against CREB, followed by a 2 h-treatment with 10 μM Fsk. (B) Progesterone secreted by MA-10 Leydig cells treated for 4 h with either DMSO or 10 μM Fsk was quantified by ELISA 2 days post-transfection of either control siRNAs (Ctrl) or siRNAs against CREB. An asterisk indicates a statistically significant difference.

Collective contribution of MEF2, CREB, and AP1 transcription factors to the regulation of Ca²⁺-dependent *Nr4a1* expression

The induction kinetic of the immediate early response gene *Nr4a1* is correlated with the chromatin state of its locus. For instance, in the renal medulla-derived PC12 cell line, the acetylation level of Lys14-H3 on the *Nr4a1* gene was shown to be

increased 1 h following cAMP stimulation, while decreasing to basal levels 3 h later (Maruoka *et al.* 2010). This explains, at least in part, the peak in *Nr4a1* expression levels at 2 h post-Fsk treatment before decreasing to basal levels at 4 h (Martin *et al.* 2008, Martin *et al.* 2009). Such promoter changes require an active modulation of acetylase (co-activator) and deacetylase (co-repressor) recruitment following cell stimulation, and this is ubiquitously mediated via interactions with transcription factors because acetylases and deacetylases do not directly bind to DNA. For instance, the transcriptional activity of MEF2 factors is modulated by interactions with co-activators and co-repressors depending on the cellular state in various tissues (Lu *et al.* 2000, McKinsey *et al.* 2000, Youn & Liu 2000, Youn *et al.* 2000). The activation of MEF2 transactivation properties by CAMKII/IV occurs by a de-repression mechanism that displaces the co-repressors HDAC4/5 from their promoter targets (Lu *et al.* 2000, McKinsey *et al.* 2000, Passier *et al.* 2000, Linseman *et al.* 2003), thus allowing a permissive transcriptional state and recruitment of the p300 acetylase leading to histone acetylation.

Inhibition of PKA was found to abrogate the cAMP-mediated increase in Lys14-H3 acetylation at the *Nr4a1* promoter (Maruoka *et al.* 2010). Furthermore, it has been shown that the cAMP pathway enhances RyR-dependent Ca²⁺ release, which occurs most likely through PKA-dependent phosphorylation of RyRs (Takasago *et al.* 1989, Yoshida *et al.* 1992, Kumar *et al.* 1994, Igami *et al.* 1999, Costa *et al.* 2010, Costa *et al.* 2011). Taking all these into consideration, it is likely that the Ca²⁺ pathway might also modulate the chromatin state at the *Nr4a1* gene promoter in Leydig cells, in addition to the general cAMP-CREB-CBP/p300 cascade. This is supported by our current results showing that pCREB and the p300 acetylase are recruited to the *Nr4a1* promoter as early as 30 min following Fsk stimulation, but this recruitment is prevented when the Ca²⁺ signaling pathway is disrupted. In addition to the ability of CREB to recruit co-activators, MEF2 along with other co-factors like NFAT can increase p300 recruitment to the *Nr4a1* promoter, as shown in T cells (Youn *et al.* 2000).

It is likely that one of the roles of MEF2 transcription factors is to constitutively silence gene expression in the absence of external stimuli, thus only allowing a transient increase when needed, similar to the dynamic profile of *Nr4a1* expression as shown in neural precursor PC12 and in MA-10 Leydig cells (Martin *et al.* 2008, Lam *et al.* 2010, Maruoka *et al.* 2010). This strategy not only helps the cell conserve energy but also avoids the initiation of any cellular process that might alter the cell basal physiology, like differentiation and steroidogenesis (Martin *et al.* 2008, Maruoka *et al.* 2010), or cause cell death [(Bouzas-Rodriguez *et al.* 2012) and reviewed in

(Deutsch *et al.* 2012)]. As MEF2 is associated with co-repressors such as HDACs on the *Nr4a1* gene promoter in unstimulated cells, it indirectly prevents unnecessary cellular processes to take place. Thus any physiological stimulus that might alter the balance of co-repressor and co-activator recruitment to the *Nr4a1* gene promoter most likely changes the interaction profile between MEF2 and its partners, including HDAC4/5 and p300. In such case, MEF2 can act as a switch allowing a transient transactivation of *Nr4a1* gene expression, and thus *Star* expression and steroidogenesis, similar to the transient elevation of intracellular cAMP and Ca²⁺ levels following trophic hormone stimulation of Leydig cells.

Several signaling pathways converge to modulate *Nr4a1* expression

In addition to the cAMP-PKA-CREB pathway, the protein kinase C (PKC) has also been shown to regulate *Nr4a1* expression in T cells by targeting the AP1/CRE elements located at -255 bp (Kim *et al.* 2005). This occurs most likely through cJUN because PKC regulates its transactivation through ATF2 in a p300-dependent manner (Yamasaki *et al.* 2009). Interestingly, the cJUN N-terminal kinase (JNK) can also activate the transactivation activity of cJUN in a Ca²⁺-dependent manner in immune cells (Su *et al.* 1994), in agreement with our results in Leydig cells. It is known that the trophic hormone stimulation of G-protein-coupled receptors increases the intracellular levels of cAMP, Ca²⁺, and DAG/IP₃ to amplify its signaling properties through PKA/CAMK/PKC. Interestingly, all of the pathways appear to converge on specific transcription factors, thus allowing proper expression/activity of the factors involved in a given cellular process, such as steroidogenesis.

In conclusion, this study suggests that, in addition to the well-known cAMP pathway, the Ca²⁺ signaling pathway activates *Nr4a1* gene transcription partially through AP1, CREB, and MEF2 transcription factors that will ultimately recruit the ubiquitous co-activator p300 to the promoter. In addition, these results convey that the cAMP and Ca²⁺ pathways converge to modulate *Nr4a1* expression and ultimately hormone-induced steroidogenesis in MA-10 Leydig cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

J J T holds a Chercheur-Boursier Scholarship from the Fonds de recherche du Québec – Santé. This work was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (262224 to JJT).

Author contribution

H S A and N M R performed the research. H S A, N M R, and J J T analyzed the data. H S A and J J T designed the research study and wrote the manuscript.

Acknowledgments

The authors thank Dr Mario Ascoli for generously providing the MA-10 cell line used in this study, Dr Dany Chabos for the cJUN expression vector, Dr Marc Montminy for the CREB expression vector, and François Péloquin for generating the *Nr4a1* promoter construct mutated in all three AP1/CRE elements.

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Received in final form 25 November 2015

Accepted 8 December 2015

Accepted Preprint published online 8 December 2015