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Evaluation of the MA-10 cell line as a model of *insl3* regulation and Leydig cell function

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ABSTRACT

Cryptorchidism, the failure of one or both testes to descend to an extra-abdominal location during mammalian development, is a common reproductive malady that often requires surgical intervention to remedy. Leydig cells are responsible for producing insulin-like peptide 3 (INSL3), a peptide hormone that is essential for normal testicular descent. The *insl3* promoter in Leydig cells can be activated by cAMP through the transcription factor Nur77, which also regulates the promoters of the steroidogenic enzymes, *cyp17* and *3β-hsd*. While the mechanism of LH action on testosterone production is well characterized, the effect of LH on *insl3* abundance has not been described. The MA-10 Leydig cell line was used to test the hypothesis that abundance of *insl3* mRNA is increased by LH/CG *via* the cAMP pathway. Cells treated with hCG had a transient robust increase in abundance of *nur77* mRNA, while *insl3* mRNA abundance remained unchanged. Further, while cAMP addition increased *nur77* mRNA abundance, it failed to affect *insl3* mRNA. Inhibition of LH-receptor-linked signal transduction pathways in the presence of hCG implicated multiple signaling networks in the regulation of both the *insl3* and *nur77* genes. Treatment with hCG and cAMP also increased abundance of *cyp17* mRNA but not *3β-hsd*. Abundance of *insl3* mRNA was not affected by hCG, testosterone or the combination of hCG and testosterone. Collectively, these results provide support for the constitutive regulation of *insl3* mRNA abundance in the MA-10 Leydig cell line rather than acute regulation by LH/CG and cAMP.

1. Introduction

Cryptorchidism is a developmental disorder characterized by failure of one or both testes to descend to an extra-abdominal location. Cryptorchidism can be detrimental to fertility in males and can lead to complications in normal castration procedures in domesticated animals, requiring more extensive surgical intervention than routine castration. Insulin-like peptide 3 (INSL3), a peptide hormone produced by the Leydig cells of the testes, is an important mediator of testicular descent as evidenced by functional studies in mice (Zimmermann et al., 1999; Adham et al., 2002). Though the central role of INSL3 in normal sexual development is acknowledged, the factors that regulate INSL3 production have yet to be comprehensively defined.

Secretion of INSL3 is a marker of Leydig cell differentiation and maturation rather than acute regulation (reviewed by Ivell et al., 2014). Evidence from clinical observations and intervention also indicates a strong correlation between LH and INSL3 even over relatively short-term treatment periods, possibly indicating a more direct relationship between gonadotropins and INSL3 (Trabado et al., 2014). Whether directly or indirectly, LH is widely considered as the secreteagogue for INSL3 though the mechanisms

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connecting LHR activation to *insl3* activation are not well understood. Activation of the cAMP/PKA pathway has been implicated, though LHR activation has also been associated with several other signal transduction pathways (Kühn and Gudermann, 1999; Salvador et al., 2002; Robert et al., 2006; Evaul and Hammes, 2008). Several transcription factors have been implicated in regulating the *insl3* promoter *in vitro*, including Nur77, KLF6, SF1 and COUP-TFII, though the complete mechanism of regulation of *insl3* mRNA production in Leydig cells remains unclear (Zimmermann et al., 1998; Martin and Tremblay, 2005; Robert et al., 2006; Mendoza-Villarroel et al., 2014; Tremblay et al., 2016).

The murine MA-10 Leydig cell line is a widely used cell model for study of Leydig cell function in mammals including most studies of *insl3* regulation (Ascoli, 1981). In the present study, there was direct testing of the hypothesis that LH functions through the cAMP/PKA pathway to increase the abundance of *insl3* mRNA in MA-10 cells.

2. Materials and methods

All reagents were purchased from Sigma Aldrich, unless otherwise indicated.

2.1. Cell culture

The MA-10 cells were generously provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Prior to plating, 25 cm^2 cell culture flasks (Corning) were incubated with 0.1% gelatin solution (0.5 g DIFCO bactogelatin, 500 ml Ca^{2+}/Mg^{2+}-free PBS) covering the growth area of the flask for 45 min at room temperature. The gelatin solution was aspirated before plating. Cells were maintained at 37 °C in 5% CO_2 in DMEM F12 growth medium (pH 7.7) supplemented with 20 mM HEPES, 15% horse serum (Invitrogen), 1% penicillin/streptomycin and 1% amphotericin B. Within each experiment, cells were cultured with horse serum from the same sample lot.

Human CG (Cell Sciences), 8-bromo cAMP, $(Bu)_2$-cAMP and testosterone were included in media at concentrations indicated in figure legends. Based on supplier recommendations, fresh preparations of hCG were obtained for each individual experiment. For signal transduction pathway inhibitor experiments, MA-10 cells were pre-treated with one of the following inhibitors for 3 h: 10 μM H 89-dihydrochloride (H89; PKA inhibitor), 10 nM wortmannin (WORT; PKB inhibitor), 10 μg/ml pseudohypericin (PH; PKC inhibitor) or 50 μM PD90859 (PD; MAPK inhibitor)(Santa Cruz Biotechnology). The pre-treatment growth media was then replaced with growth media containing either 0 or 100 ng/ml hCG and the inhibitor for an additional 1 or 3 h before RNA extraction, as indicated in Fig. 2A, B.

2.2. RNA extraction and reverse transcription

Cells were released from flasks through incubation with 0.5X trypsin/EDTA solution and subsequently washed in 5 ml media by centrifugation. Total RNA was extracted from the resulting cell pellet using the RNeasy® Mini Kit (Qiagen). The RNA concentration of each sample was determined by spectrophotometry. First strand cDNA was synthesized from 1 μg RNA using the iScript™ Select cDNA Synthesis Kit (BioRad), with oligo dT primers.

2.3. Quantitative real time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was used to quantify target gene mRNA abundance. Gene-specific primers (Table 1) were used to amplify target genes in cDNA samples using an Applied Biosystems 7500 Fast Real-Time PCR System (one cycle at 95 °C for 20 s, 50 cycles at 95 °C for 3 s, and one cycle at 60 °C for 30 s) with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The specificity of each primer set was verified by dissociation curve. To further validate the *insl3* primers, the qPCR product was gel purified using the Qiagen Qiaquick® Gel Extraction Kit per the manufacturer's protocol and sequenced by Sequetech (Mountain View, CA). Primer efficiencies for all primer sets used were calculated from Ct values using serial dilutions and determined to be within the acceptable range of 90% to 110% prior to use in experiments. Target mRNA abundance values were obtained using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the abundance of TATA binding protein (TBP) mRNA as an internal control to correct for reverse transcription efficiency. Initially, beta-actin, RNA polymerase III, ribosomal protein L6 and TBP were evaluated as potential internal control genes, though only TBP exhibited stable expression across all treatment conditions ($P > 0.7$). All reactions

Table 1
Gene specific primers for quantitative polymerase chain reaction.

Gene		Primer sequence (5'-3')	Product Size	Accession number
Mus musculus insl3	Sense Antisense	CCTCCTGGCTATGTCATTGCAACA CTGTGGTCCTTGCTTACTGCGAT	85bp	NM_013564.6
Mus musculus nur77	Sense Antisense	AGACCTGTTGCTAGAGTCTGCCTT TCAATCCAATCACCAAAGCCACGG	147bp	NM_010444.2
Mus musculus cyp17	Sense Antisense	TGCTCATTCCACACAAGGCTAACA TAAACCGATCTGGCTGGTCCCATT	132bp	NM_007809.3
Mus musculus 3β-hsd1	Sense Antisense	TCAGCCACCACCATCTCAGACTTT AGCCGCTCAGTTCAGAATGTAGGA	81bp	NM_008293.3
Mus musculus tbp	Sense Antisense	ACACTCAGTTACAGGTGGCAGCAT CAAGTAGCAGCACAGAGCAAGCAA	130bp	NM_013684

Accession numbers indicate the GenBank sequence that was referenced to design primers using IDT DNA Technologies PrimerQuest software; Primers were validated and chosen based upon their scores in both NetPrimer and Beacon Designer software programs.

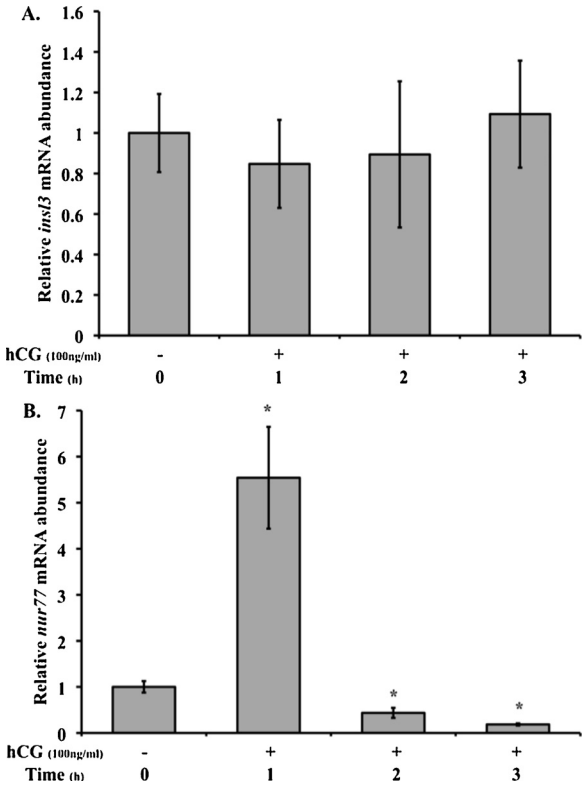

Fig. 1. Effect of hCG on *insl3* and *nur77* mRNA abundance; *Insl3* (A) and *nur77* (B) mRNA abundance in MA-10 cells treated with 100 ng/ml hCG for 1, 2 and 3 h; Values represent the mean ± SEM ($n = 3$); an asterisk indicates a difference from the control ($P < 0.05$).

were performed in duplicate for quality control and repeated if the coefficient of variation from the calculated abundance (2^{-Ct}) exceeded 20% between the technical replicates.

2.4. Statistical analysis

All qPCR data were analyzed using the general linear model in Statistical Analysis Software (SAS Carey, NC), with all treatments being compared to the control. The abundance of target mRNA is reported as the mean ± SEM (n = 3). Means were considered significantly different at $P < 0.05$

3. Results

Treatment with 100 ng/ml hCG did not have an effect on *insl3* mRNA abundance at 1, 2, or 3 h (Fig. 1A). The capacity of MA-10 cells to respond to gonadotropin stimulation was confirmed by measuring *nur77* mRNA abundance in hCG-treated samples. There was a significant but transient increase in *nur77* mRNA abundance at 1 h of treatment, while at 2 and 3 h, *nur77* mRNA abundance was less than that of the control (Fig. 1B).

To determine which signal transduction pathway the LHR uses to regulate *nur77* mRNA abundance in the Leydig cell, MA-10 cells were subjected to a 3 h pre-treatment period with inhibitors of four pathways that have been shown to be utilized by the LHR: cAMP/PKA, PI3K/PKB, PKC and MAPK signal transduction pathways. Following pre-treatment, the cells were treated with media containing the inhibitor with which the sample was pre-treated and 100 ng/ml hCG for 1 and 3 h. The abundance of *insl3* mRNA was not increased in response to hCG alone. Inhibition of any one pathway did not affect *insl3* mRNA abundance, though there was an increase in *insl3* mRNA abundance when the PI3K/PKB pathway was inhibited in the presence of hCG for 3 h (Fig. 2A). The abundance of *nur77* mRNA was increased in response to hCG treatment for 1 h as described previously, though this induction was not affected by the addition of any single pathway inhibitor (Fig. 2B). The pre-treatment period alone did not affect *insl3* or *nur77* mRNA abundance (data not shown).

Though cAMP increases *insl3* mRNA in the MA-10 cell line (Robert et al., 2006), inhibition of the cAMP/PKA pathway with or without hCG treatment did not affect the abundance of *insl3* mRNA in the MA-10 cell line (Fig. 2A). Treatment with $(Bu)_2$-cAMP for 6 h decreased *insl3* mRNA abundance, while 8-bromo cAMP did not affect *insl3* mRNA abundance (Fig. 3A), though both cAMP analogs increased *nur77* mRNA abundance after 6 h of treatment (Fig. 3B). Because cAMP is a second messenger and, therefore, may have an effect in a shorter time frame, cells were treated with 0.5 mM $(Bu)_2$-cAMP for 0.5, 1, 2 and 4 h. The abundance of *insl3* mRNA

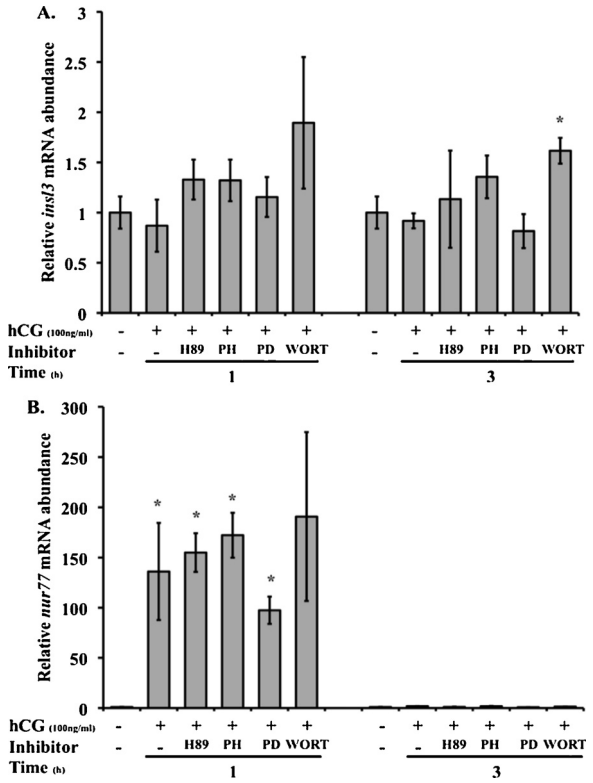

Fig. 2. Effect of hCG and pathway inhibition on *insl3* and *nur77* mRNA abundance; *Insl3* (A) and *nur77* (B) mRNA abundance in MA-10 cells treated with 10 μM H 89-dihydrochloride (H89; PKA inhibitor), 10 nM wortmannin (WORT; PKB inhibitor), 10 μg/ml pseudohypericin (PH; PKC inhibitor) or 50 μM PD90859 (PD; MAPK inhibitor) and 100 ng/ml hCG for 1 and 3 h; Values represent the mean ± SEM ($n = 3$); an asterisk indicates a difference from the control ($P < 0.05$).

was still unaffected, even at these shorter time points (Fig. 4A), while *nur77* mRNA abundance was increased at all time points (Fig. 4B).

To assess the status of mRNA encoding some key steroidogenic enzymes in the MA-10 cell line, the abundance of *3β-hsd* and *cyp17* mRNA was quantified in response to hCG and cAMP. Neither 100 ng/ml hCG or 0.5 mM cAMP at any time point had any effect on *3β-hsd* mRNA abundance (Fig. 5A, C). The abundance of *cyp17* mRNA, however, increased in response to 100 ng/ml hCG at 1, 2, and 3 h and 0.5 mM $(Bu)_2$-cAMP at 2 and 4 h (Fig. 5B, D).

The capacity of this cell line to respond to gonadotropin stimulation was confirmed by the increase in *nur77* mRNA abundance in response to hCG. Others have reported that testosterone increases *insl3* mRNA abundance in the MA-10 cell (Lague and Tremblay, 2008). Because this cell line has been reported to lack full CYP17 activity and therefore produces progesterone instead of testosterone (Hoelscher and Ascoli, 1996), the potential autocrine actions of testosterone on *insl3* mRNA production would not be apparent in the MA-10 cell line. The MA-10 cells, therefore, were supplemented with exogenous testosterone. While testosterone alone did not affect *nur77* mRNA abundance, hCG alone increased *nur77* mRNA abundance at both 1 and 3 h. Additionally, the combination of hCG and testosterone increased *nur77* mRNA abundance at both 1 and 3 h (Fig. 6A). In accordance with the initial treatment of MA-10 cells with hCG, *insl3* mRNA abundance remained unchanged at all time points. Interestingly, treatment of MA-10 cells with 15 nM testosterone did not affect *insl3* mRNA abundance, nor did the combination of hCG and testosterone (Fig. 6B).

4. Discussion

Though there is evidence in the literature implicating LH as a direct stimulus for *insl3* gene transcription stimulation, treatment with hCG did not affect *insl3* mRNA abundance in the MA-10 cell line. These data could indicate either a lack of capacity of the MA-10 cell to respond to gonadotropin stimulation or that there are other means by which the *insl3* gene is regulated. The robust increase in *nur77* mRNA abundance in response to hCG indicates that these cells are indeed capable of responding to gonadotropin stimulation, suggesting an alternative mechanism responsible for regulating *insl3* mRNA abundance in the Leydig cell, or bolstering the notion that INSL3 production is not acutely regulated but rather a constitutive marker of Leydig cell maturity (Ivell et al., 2014).

Additional evidence to support an alternative stimulus of *insl3* gene transcription stimulation comes from the observation that individual inhibition of the LHR-linked cAMP/PKA, PKC or MAPK pathways in the presence of hCG did not have an effect on *insl3* mRNA abundance at 1 or 3 h. The exception is the significant increase in *insl3* mRNA abundance observed in the presence of hCG and

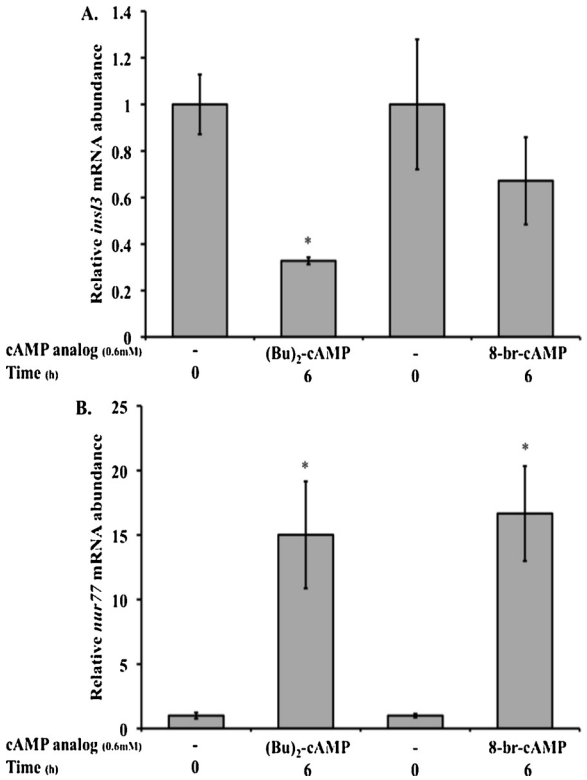

Fig. 3. Effect of cAMP analogs on *insl3* and *nur77* mRNA abundance; Insl3 (A) and *nur77* (B) mRNA abundance in MA-10 cells treated with 0.6 mM 8-bromo cAMP or 0.6 mM $(Bu)_2$-cAMP for 6 h; Values represent the mean ± SEM ($n = 3$); an asterisk indicates difference from the control ($P < 0.05$).

wortmannin for 3 h, indicative of hCG being capable of inducing *insl3* mRNA after 3 h, but only when the PI3K/PKB pathway is inhibited. Multiple pathways could be responsible for activating or repressing the *insl3* gene. These data indicate that the PI3K/PKB pathway may have a suppressive effect on *insl3* mRNA. Individually, the cAMP/PKA, PKC or MAPK kinase pathways do not seem to have a function in *insl3* mRNA abundance regulation, though these pathways may function in concert with other pathways that were not assessed in the present study. Inhibition of multiple pathways at once may provide further insight into the combined actions these pathways in regulating the production of *insl3* mRNA.

Though hCG treatment for 1 h consistently increased *nur77* mRNA abundance, the pathway through which hCG function to produce this effect remains unclear. The addition of any single pathway inhibitor did not affect the capacity of hCG to induce *nur77* mRNA production. As with *insl3* mRNA, these data indicate that there may be multiple pathways that regulate *nur77* mRNA abundance in the Leydig cell, potentially including pathways that were not included in this experiment. A similar experiment was conducted in the K28 Leydig cell line, where cells were treated for 1 h with 200 ng/ml LH and either 10 u M H89 (PKA inhibitor), 100 nM bisindolylmaleimide I (PKC inhibitor) or 10 nM wortmannin (PKB inhibitor) (Song et al., 2001). *Nur77* mRNA abundance was assessed by Northern blot and LH treatment alone increased *nur77* mRNA abundance, consistent with results from the present study. When PKA was inhibited in the presence of LH, however, the LH- mediated induction of *nur77* gene transcription stimulation was decreased by 73%. Further, inhibition of PKC and PKB in the presence of LH resulted in a 76% and 70% reduction in *nur77* mRNA abundance, respectively (Song et al., 2001). It is important to note that in the present work, the same PKA and PKB inhibitors were utilized at the same concentration, with results not being consistent with those from previous studies. Though an effect of pathway inhibition was observed in the K28 Leydig cell line and not in the present study in the MA-10 cell line, what is consistent is the indication that multiple pathways regulate *nur77* mRNA abundance in the Leydig cell.

Inhibition of the cAMP/PKA pathway did not affect *insl3* or *nur77* mRNA abundance. Similarly, treatment of MA-10 cells with cAMP analogs did not increase *insl3* mRNA abundance; specifically, treatment with 0.5 mM $(Bu)_2$-cAMP for 4 h failed to increase *insl3* mRNA abundance. These results are inconsistent with those of Robert et al. (2006), though the same cell line and experimental design was utilized. Interestingly, though both cAMP analogs at all time points increased *nur77* mRNA, inhibition of the cAMP/PKA pathway did not abrogate the hCG-induced increase in *nur77* mRNA abundance. While the cAMP-mediated increase in *nur77* mRNA implicates the cAMP/PKA pathway in *nur77* gene transcription stimulation, the inhibition of that pathway does not corroborate those results, suggesting that the cAMP/PKA pathway may not be the only pathway involved in the hCG-regulation of *nur77* mRNA abundance.

The abundance of 3β-hydroxysteroid dehydrogenase (*3β-hsd*) mRNA remained unchanged in the presence of hCG and cAMP. Though 3β-HSD activity, mRNA and protein abundance increase in primary Leydig cell cultures in response to LH and cAMP, these

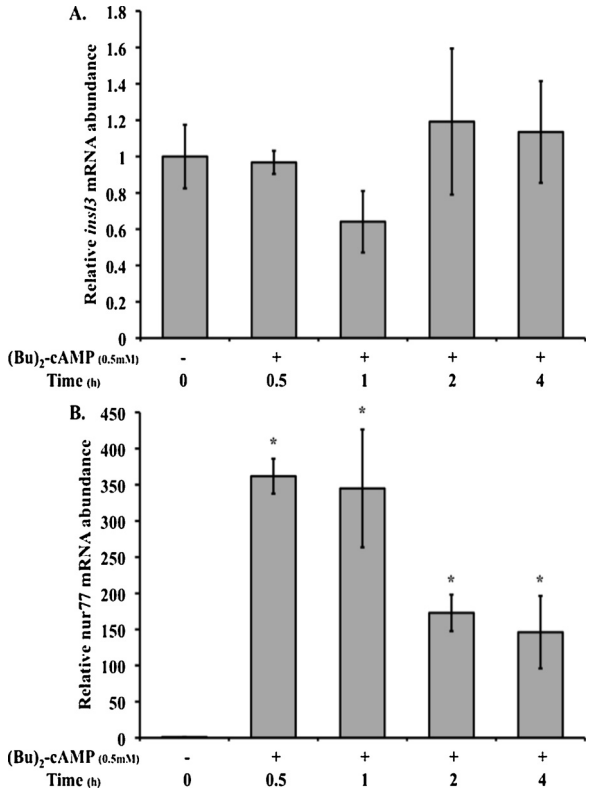

Fig. 4. Effect of $(Bu)_2$-cAMP on *insl3* and *nur77* mRNA abundance at four different time points; Insl3 (A) and *nur77* (B) mRNA abundance in MA-10 cells treated with 0.5 mM $(Bu)_2$-cAMP for 0.5, 1, 2, and 4 h (C); Values represent the mean ± SEM ($n = 3$); an asterisk indicates a difference from the control ($P < 0.05$).

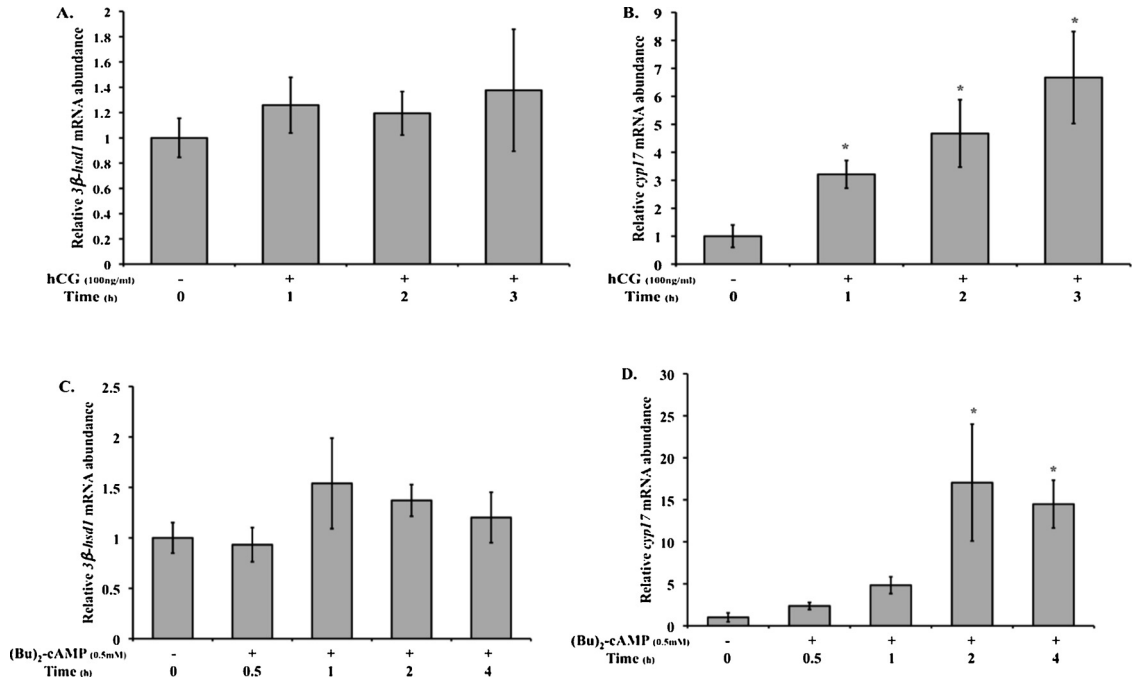

Fig. 5. Effect of hCG and cAMP on *3β-hsd* and cyp17 mRNA abundance; *3β-hsd* and *cyp17* mRNA abundance in MA-10 cells treated with 100 ng/ml hCG for 1, 2 and 3 h (A,C) or 0.5 mM $(Bu)_2$-cAMP for 0.5, 1, 2 and 4 h (B,D); Values represent the mean ± SEM ($n = 3$); an asterisk indicates a difference from the control ($P < 0.05$).

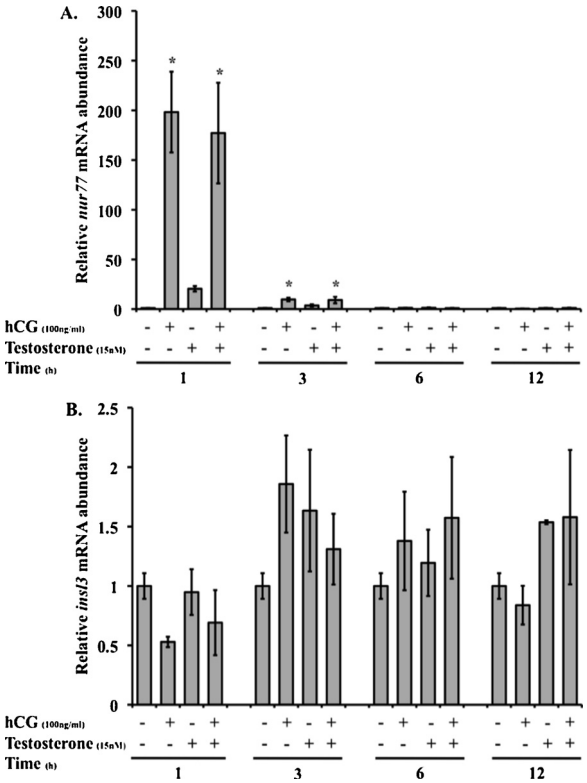

Fig. 6. Effect of hCG and testosterone on *insl3* and *nur77* mRNA abundance; *Insl3* (A) and *nur77* (B) mRNA abundance in MA-10 cells treated with 100 ng/ml hCG and 15 nM testosterone in a 2 × 2 factorial design for 1 and 3 h; Values represent the mean ± SEM ($n = 3$); an asterisk indicates a difference from the control ($P < 0.05$).

effects were not evident until 24 h of treatment (Keeney and Mason, 1992), indicating the effects of hCG and cAMP on *3β-hsd* mRNA abundance may not be observed until a later time point than those assessed in the current study. Additionally, in primary Leydig cell cultures, *3β-hsd* mRNA has been shown to be in relatively greater abundance in the absence of either LH or cAMP (Payne and Sha, 1991). The induction of the *cyp17* gene in the presence of both hCG and cAMP is consistent with previous results in primary mouse Leydig cell studies, which indicated that CYP17 enzyme activity was basically undetectable in the absence of LH or cAMP, but when cells were treated with LH or cAMP, there was a time-dependent increase in CYP17 activity over the course of 5–7 days (Malaska and Payne, 1984; Rani and Payne, 1986; Payne and Youngblood, 1995). The present data indicate that a time-dependent increase in *cyp17* mRNA may be observed over a longer period of time than assessed in this study.

Treatment of MA-10 cells with testosterone for 36 h increases *insl3* mRNA abundance (Lague and Tremblay, 2008). In the present study, 12 h of treatment of MA-10 cells with testosterone or the combination of hCG and testosterone did not affect *insl3* mRNA abundance. Testosterone may still have a function in *insl3* production through an autocrine action on the Leydig cell, though perhaps over a longer period of time. Treatment with hCG and the combination of hCG and testosterone significantly increased *nur77* mRNA abundance at 1 and 3 h of treatment. The primary stimulus for this increase seems to be hCG, because testosterone treatment alone did not have an effect and hCG treatment alone resulted in an increase similar to that observed with the combination of hCG and testosterone.

The Nur77 protein is a downstream effector of the cAMP/PKA pathway and must be dephosphorylated at several serine residues to allow and promote the binding of this transcription factor to the promoters that it regulates (Hirata et al., 1993; Maira et al., 2003; Tremblay et al., 2009). The specific mechanism by which this dephosphorylation is regulated and the identity of the protein phosphatase that modulates this activation of Nur77, however, remains to be identified. If the MA-10 cell line has lost the capacity to synthesize the protein phosphatase responsible for activating Nur77, hCG and cAMP may not have the capacity to induce an increase *insl3* mRNA because of the lack of Nur77 activation.

The presence of a repressor that interacts with the *insl3* promoter could also account for lack of induction of *insl3* in the presence of both cAMP and hCG. In this case, if the stimulus required to remove the repressor is not expressed by the MA-10 cell line, the repressor would continue to inhibit transcription and the mRNA abundance of *insl3* would not increase in response to cAMP or hCG. Though a repressor that specifically regulates *insl3* production has not been identified, inhibition of Nur77 action may also have an effect on *insl3* mRNA abundance. The androgen receptor corepressor-19 kDa (ARR19) inhibits the transactivation of Nur77 in MA-10 cells by interacting with Nur77 and thereby prevents Nur77 from binding to the coactivator steroid receptor coactivator (SRC-1). This was ascertained by transfecting MA-10 cells with Nur77 response element-driven reporter vectors and Nur77 and ARR19 expression

vectors. In this context, the presence of ARR19 resulted in the repression of Nur77 transactivation in a dose-dependent manner. Furthermore, when MA-10 cells were co-transfected with CYP17, StAR and 3β-HSD reporter constructs and Nur77 and ARR19 expression vectors, ARR19 inhibited Nur77-induced promoter activity of each of the tested steroidogenic enzyme genes. Furthermore, the ARR19 protein regulates Nur77 transactivation by physically interacting with this transcription factor and preventing Nur77 from binding SRC-1 (Qamar et al., 2010).

5. Conclusions

The MA-10 cells are functionally capable of responding to gonadotropin stimulation and have been considered to be a useful model to study regulation of the *insl3* gene. Results from the present study indicate that while the MA-10 cell line is responsive to gonadotropin stimulation through *nur77* and *cyp17* stimulation of gene transcription, that response does not include acute regulation of *insl3* mRNA abundance. Data from the present study are inconsistent with previously published data in terms of *insl3* gene transcription stimulation in response to cAMP and could be interpreted as supportive for the notion that INSL3 is a constitutive product reflecting Leydig cell maturity rather than being acutely regulated by gonadotropins. Results of the present study do not negate the possibility of more direct regulation by LH, and that there may be multiple regulatory mechanisms functioning in the Nur77-regulated production of *insl3* by the MA-10 Leydig cell.

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