Blocking L-type calcium channels reduced the threshold of cAMP-induced steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells

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Abstract

Previous studies have reported the roles of Ca^{2+} in steroidogenesis. The present study has investigated an inhibitory effect of Ca^{2+} influx through L-type Ca^{2+} channels on gene expression of steroidogenic acute regulatory (STAR) protein that regulates the transfer of substrate cholesterol to the inner mitochondrial membrane for steroidogenesis. Blocking Ca^{2+} influx through L-type Ca^{2+} channels using the selective Ca^{2+} channel blocker, nifedipine, markedly enhanced cAMP-induced STAR protein expression and progesterone production in MA-10 mouse Leydig cells. This was confirmed by utilization of different L-type Ca^{2+} channel blockers. Reverse transcription-PCR analyses of *Star* mRNA and luciferase assays of *Star* promoter activity indicated that blocking Ca^{2+} influx through L-type Ca^{2+} channels acted at the level of *Star* gene transcription. Further studies showed that blocking the Ca^{2+} channel enhanced *Star*

gene transcription by depressing the expression of DAX-1 (NR0B1 as listed in the MGI Database) protein, a transcriptional repressor of *Star* gene expression. It was also observed that there is a synergistic interaction between nifedipine and cAMP. Normally, sub-threshold levels of cAMP are unable to induce steroidogenesis, but in the presence of the L-type Ca^{2+} channel blocker, they increased STAR protein and steroid hormone to the maximal levels. However, in the absence of minimal levels of cAMP, none of the L-type Ca^{2+} channel blockers are able to induce *Star* gene expression. These observations indicate that Ca^{2+} influx through L-type Ca^{2+} channels is involved in an inhibitory effect on *Star* gene expression. Blocking L-type Ca^{2+} channel attenuated the inhibition and reduced the threshold of cAMP-induced *Star* gene expression in Leydig cells.

Journal of Endocrinology (2010) **204**, 67–74

Introduction

Steroidogenesis in testicular Leydig cells is mainly regulated by LH secreted from the pituitary gland. Binding of LH to its receptor on Leydig cells induces cAMP-dependent signaling and cAMP-independent signaling. These signaling pathways act in concert to regulate steroidogenesis through their effects on the expression or activity of steroidogenic proteins in Leydig cells (Payne & Youngblood 1995, Cooke 1999, Wang & Stocco 1999).

In these signaling pathways, Ca^{2+} -mediated signaling has been reported to play an important role in steroidogenesis. LH or human chorionic gonadotropin (hCG) stimulation of Leydig cells increases Ca^{2+} influx (Sullivan & Cooke 1986, Kumar *et al.* 1994). In the absence of Ca^{2+} , LH- or cAMP-stimulated testosterone production is reduced. Also, the reduced testosterone levels could be restored by the addition of Ca^{2+} to the culture medium (Janszen *et al.* 1976, Ramnath *et al.* 1997).

Further studies suggest that Ca^{2+} affect the transfer of the substrate cholesterol to the inner mitochondrial membrane, the rate-limiting step in steroidogenesis (Hall *et al.* 1981, Meikle *et al.* 1991). This was confirmed by a study reporting a Ca^{2+} -induced increase in steroidogenic acute regulatory (STAR) protein (Manna *et al.* 1999), which is critical for the cholesterol transfer to the inner mitochondrial membrane to initiate steroidogenesis (Clark *et al.* 1994, Lin *et al.* 1995, Wang *et al.* 1998). While hCG-induced STAR protein expression and steroid production are enhanced by Ca^{2+} , the increases in STAR protein and steroid hormone are reversed by calcium chelators. In addition, the effect of Ca^{2+} on hCG-induced *Star* mRNA expression is suppressed by blocking L-type Ca^{2+} channels using a blocker, verapamil (Manna *et al.* 1999).

However, observations on the roles of L-type Ca^{2+} channels in *Star* gene expression and steroidogenesis were not consistent in the previous studies. While blocking Ca^{2+} influx through L-type Ca^{2+} channels attenuates the progesterone production enhanced by the flavonoid,

quercetin, it does not affect the increase in *Star* promoter activity in MA-10 mouse Leydig cells (Chen *et al.* 2007). Also, a previous study reported that androgen production is unaffected by L-type Ca^{2+} channel blockers at concentrations <0.1 mM (Moger 1983). The data from the study show that blocking L-type Ca^{2+} channel produces a biphasic effect on steroidogenesis, with LH-induced androgen production being increased to the maximal levels and then reduced, as the concentration of verapamil in the cultures is increased. It is possible that the concentrations of L-type Ca^{2+} channel blockers, levels of tropic hormones or extra- and intra-cellular Ca^{2+} status alter the effects of Ca^{2+} influx through L-type Ca^{2+} channels on *Star* gene transcription and steroid production. It was also observed that Ca^{2+} is an inhibitor of hCG-activated adenylate cyclase activity in MA-10 mouse Leydig cells (Pereira *et al.* 1988). This inhibitory effect may reduce cAMP formation and attenuate hCG-stimulated *Star* gene expression. Therefore, further studies are required to understand the roles of L-type Ca^{2+} channel in *Star* gene expression. The present study has investigated an inhibitory effect generated by Ca^{2+} influx through L-type Ca^{2+} channel on *Star* gene transcription in MA-10 mouse Leydig cells.

Materials and Methods

Reagents

$\text{N}^6,2'$ -dibutyryl cAMP (dbcAMP), H89, and Waymouth's MB/752 medium were purchased from Sigma. H8 was purchased from EMD Chemicals (Gibbstown, NJ, USA). Nifedipine, verapamil, isradipine, and diltiazem were purchased from BIOMOL (Plymouth Meeting, PA, USA). Rabbit antiserum generated against STAR protein was a generous gift from Dr D B Hales (University of Illinois, Chicago; Hales *et al.* 2000). The monoclonal antibody against DAX-1 (NR0B1 as listed in the MGI Database) protein was a generous gift from Dr E Lalli (Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France). Donkey anti-rabbit IgG antibody conjugated with HRP was purchased from Biosource (Camarillo, CA, USA). Horse serum was purchased from Invitrogen. The Dual-Luciferase Reporter Assay System was purchased from Promega. Other common chemicals used in this study were obtained from either Sigma or Fisher Chemicals (Pittsburgh, PA, USA).

MA-10 cell culture

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr Mario Ascoli (University of Iowa, Iowa City). The cells were cultured in 12-well culture plates in Waymouth's MB/752 medium containing 15% horse serum as previously described (Ascoli 1981), in an incubator at 37°C and 5% CO_2 . Before each experiment, the medium was replaced with serum-free Waymouth's medium.

Steroid hormone production

MA-10 cells were cultured for 30 min in serum-free Waymouth's medium with or without L-type Ca^{2+} channel blockers (as described in the figure legends), and then 0.05 mM dbcAMP was added to the culture for 6 h. The medium was collected at the end of each experiment and stored at -20°C . The concentration of progesterone in the medium was determined by RIA (Resko *et al.* 1974).

Western blot analysis

Western blot analyses were performed to detect STAR protein and DAX-1 protein in MA-10 cells as previously described (Townson *et al.* 1996). Western blot analysis experiments were repeated at least thrice, and the results of one representative experiment are shown for each figure.

Transfections

MA-10 cells were cultured in 12-well plates, with 0.2×10^6 cells/well, overnight. The cells in each well were transfected with 0.5 μg DNA of the *Star* promoter/luciferase plasmid, PGL2/*Star*, expressing firefly luciferase driven by the -966 bp sequences of the *Star* promoter (Caron *et al.* 1997). Transfections also included 6.0 ng pRL-SV40 vector DNA (a plasmid, which constitutively expresses Renilla luciferase under the control of the SV40 promoter, Promega). Transfections were performed using FuGENE HD Transfection Reagent (Roche) following the manufacturer's instructions. After 48-h culture, the cells were utilized for further experiments.

Luciferase assays

Following experiments, the cells were washed with cold PBS and lysed with Passive Lysis Buffer (Promega). The supernatants were utilized for luciferase assays using a Dual Luciferase Reporter Assay System following the manufacturer's instructions (Promega). The Relative Light Units (RLU, determined by dividing the reading from the PGL2/*Star* promoter by the reading from Renilla luciferase) were measured using a TD-20/20 luminometer (Turner Designers, Sunnyvale, CA, USA). The *Star* promoter activities were expressed as fold over RLU of control.

Reverse transcription-PCR

Experiments were performed for reverse transcription (RT)-PCR analyses of *Star* mRNA and *Dax-1* (*Nr0b1*) mRNA expressions. After experiments, MA-10 cells were washed with cold PBS and used for total RNA purification using TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen). The first-strand cDNA was synthesized from the RNA using the Reverse Transcription

Figure 1 Blocking Ca^{2+} influx through L-type Ca^{2+} channel enhanced cAMP-induced steroidogenesis and STAR protein expression in MA-10 mouse Leydig cells. MA-10 cells were cultured in serum-free Waymouth's medium with increasing concentrations of the selective L-type Ca^{2+} channel blocker, nifedipine, for 30 min, and then 0.05 mM dbcAMP was added to the culture for 6 h. (A) The cells were collected, and 20 μg cell lysate protein was used for western blot analysis of STAR protein. (B) Progesterone concentration in the medium was assessed by RIA. ** $P < 0.01$; *** $P < 0.001$; $n = 3$; compared with 0.05 mM dbcAMP alone.

System (Promega). PCR was performed as previously described for analyses of *Star* mRNA (Rao *et al.* 2003) and *Dax-1* mRNA (Jana *et al.* 2008). β -Actin was used as an internal marker.

Quantitative real-time PCR

Total RNA was prepared from MA-10 cells after experiments, and cDNA was synthesized from the RNA as described above. Real-time PCR was performed with the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), for 10 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). The primer sets for the real-time PCR of mouse *Dax-1*, *Star*, and β -actin (Applied Biosystems' Assays ID: Mm00431729_m1, Mm00441558_m1, and Mm02619580_g1 respectively) were chosen from the collection of the TaqMan Gene Expression Assays (Applied Biosystems). The method of relative quantitation was used for the real-time PCR following the manufacturer's instructions.

Statistical analysis

Each experiment was repeated at least thrice. Statistical analysis of the data was performed with ANOVA followed by Tukey's significant difference test using the GraphPad Prism 4 system (GraphPad Software, San Diego, CA, USA). The data are shown as the mean $\pm$ S.E.M.

Results

Steroid hormone production

MA-10 mouse Leydig cells were incubated with increasing concentrations of the selective L-type Ca^{2+} channel blocker, nifedipine, for 30 min, followed by the addition of 0.05 mM dbcAMP for 6 h to determine the effect of Ca^{2+} influx through L-type Ca^{2+} channels on steroidogenesis. The stimulation with 0.05 mM dbcAMP alone did not induce significant increase in steroidogenesis in MA-10 mouse Leydig cells, but co-incubation with nifedipine significantly increased the progesterone production in a concentration-dependent manner. Progesterone concentration in the culture medium was increased from 9.4 to 97.9 ng/ml, as nifedipine in the culture was increased from 0 to 50 μM (Fig. 1).

STAR protein expression

Western blot analyses were performed to determine whether blocking Ca^{2+} influx through L-type Ca^{2+} channels affects STAR protein expression in MA-10 cells. As shown in Fig. 1, when L-type Ca^{2+} channel was blocked with the increasing concentrations of nifedipine, STAR protein expression was markedly elevated in MA-10 cells cultured in the medium containing 0.05 mM dbcAMP. The increase in STAR protein expression also occurred in a concentration-dependent manner, which was concomitant with the increase in progesterone production.

Figure 2 Effects of different L-type Ca^{2+} channel blockers on cAMP-induced steroidogenesis and STAR protein expression in MA-10 mouse Leydig cells. MA-10 cells were cultured in serum-free Waymouth's medium with 25 μM each of the L-type Ca^{2+} channel blockers, verapamil, isradipine, and diltiazem respectively for 30 min, and then 0.05 mM dbcAMP was added to the culture for 6 h. (A) The cells were collected and 20 μg cell lysate protein was used for western blot analysis of STAR protein. (B) Progesterone concentration in the medium was assessed by RIA. *** $P < 0.001$; $n = 4$; compared with 0.05 mM dbcAMP alone.

The increases in STAR protein expression and steroidogenesis that resulted from the blocking of L-type Ca^{2+} channels were confirmed by the utilization of different L-type Ca^{2+} channel blockers, including verapamil, isradipine, and diltiazem. Co-incubation of each blocker with 0.05 mM dbcAMP dramatically increased STAR protein expression and steroid hormone production in MA-10 cells. Progesterone production was increased by 7.3-, 5.9-, and 4.6-fold respectively by verapamil, isradipine, and diltiazem over that induced by 0.05 mM dbcAMP alone (Fig. 2).

Star gene transcription

Luciferase assays of *Star* promoter activity and RT-PCR analyses of *Star* mRNA levels were performed to understand the mechanism responsible for the inhibitory effect of Ca^{2+} influx through L-type Ca^{2+} channels on STAR protein expression. Incubation of MA-10 cells with increasing levels of nifedipine resulted in a concentration-dependent increase in *Star* promoter activity. The *Star* promoter activity increased 2.6-fold over controls when the concentration of nifedipine in the cultures was increased to 50 μM . Similarly, the results

Figure 3 Blocking Ca^{2+} influx through L-type Ca^{2+} channel enhanced cAMP-induced *Star* gene transcription in MA-10 mouse Leydig cells. MA-10 cells were cultured in serum-free Waymouth's medium with increasing concentrations of the selective L-type Ca^{2+} channel blocker, nifedipine, for 30 min, and then 0.05 mM dbcAMP was added to the culture for 6 h. (A) The cells were collected for total RNA isolation, and *Star* mRNA was analyzed by RT-PCR with β -actin as an internal marker. (B) MA-10 cells were transfected with a *Star* promoter/luciferase plasmid (PGL2/*Star*) and a pRLSV40 vector, a plasmid that constitutively expresses Renilla luciferase. The cells were then treated as described above, and the cell lysate was used for luciferase assays using a Dual-Luciferase Reporter Assay System. The Relative Light Unit (RLU) was determined by dividing the reading from the PGL2/*Star* promoter by the reading from Renilla luciferase. The promoter activities were expressed as fold over RLU of control. *** $P < 0.001$, $n = 3$; compared with 0.05 mM dbcAMP alone.

Figure 4 Synergistic interaction between nifedipine and cAMP in steroidogenesis of MA-10 mouse Leydig cells. MA-10 cells were cultured for 30 min in serum-free Waymouth's medium with or without 20 μM nifedipine, and then increasing concentrations of dbcAMP were added to the cultures for 6 h. (A) The cells were collected, and 20 μg cell lysate protein was used for western blot analysis of STAR protein. (B) Progesterone production in the culture medium was assessed by RIA. * $P < 0.05$; *** $P < 0.001$; $n = 3$; compared with the paired group stimulated with dbcAMP alone.

obtained from RT-PCR analyses of *Star* mRNA showed that *Star* mRNA levels were increased as the levels of nifedipine in the cultures increased (Fig. 3).

Synergistic interaction between nifedipine and cAMP

To examine the interaction between cAMP and nifedipine, MA-10 cells were treated with or without 20 μM nifedipine for 30 min, and then increasing concentrations of dbcAMP were added to the culture medium for 6 h. The results shown in Fig. 4 demonstrate that the sub-threshold level of dbcAMP (0.05 or 0.1 mM) alone was unable to stimulate significant increases in STAR protein expression and steroidogenesis. However, in the presence of 20 μM nifedipine, these low levels of dbcAMP increased STAR protein expression and steroid hormone production. Similarly, while nifedipine itself did not increase STAR protein and steroid hormone synthesis, in the presence of sub-threshold levels of dbcAMP (0.05 or 0.1 mM), nifedipine dramatically increased STAR protein expression and progesterone production in MA-10 cells. In the cells treated with 0.1 mM dbcAMP, nifedipine increased progesterone production 6.6-fold over that of cells treated with 0.1 mM dbcAMP alone. Although blocking Ca^{2+} influx through L-type Ca^{2+} channels enhanced cAMP-induced steroidogenesis, this effect was reduced when the levels of cAMP were higher than the threshold of STAR protein expression. When dbcAMP level in the cultures increased to 0.5 mM, nifedipine failed to increase STAR protein expression and steroid production (Fig. 4).

Critical role of protein kinase A in verapamil-enhanced steroidogenesis

The role of protein kinase A (PKA) in the verapamil-enhanced steroidogenesis was investigated using the PKA inhibitor H89 or H8. While co-action of 15 μ M verapamil and 0.05 mM dbcAMP dramatically increased STAR protein, inhibition of PKA activity with either H89 or H8 reversed this increase. Similarly, while progesterone concentration was increased from 6.6 to 86.0 ng/ml by verapamil in the same culture, it was reduced to 27.4 or 40.6 ng/ml by inhibition of PKA activity using H89 or H8 respectively (Fig. 5). When 22(R)hydroxylcholesterol was added to the cultures, there was no significant difference in progesterone production among the groups, indicating that the activities of steroidogenic enzymes were not affected by the treatments.

Dax-1 gene transcription

To further understand how Ca^{2+} influx through L-type Ca^{2+} channels inhibited *Star* gene transcription, the expression of the transcriptional repressor, DAX-1 protein, was analyzed by western blot and RT-PCR with MA-10 cells treated with verapamil and 0.05 mM dbcAMP. Western blot analyses showed that verapamil markedly reduced DAX-1 protein. The results were verified by RT-PCR analyses of

Figure 5 Inhibition of protein kinase A activity reversed the verapamil-enhanced steroidogenesis and STAR protein expression. MA-10 cells were cultured in serum-free Waymouth's medium with 15 μ M of the selective L-type Ca^{2+} channel blocker, verapamil, and 15 μ M of PKA inhibitor H89 or H8 for 30 min, and then 0.05 mM dbcAMP was added to the culture for 6 h. (A) The cells were collected, and 20 μ g cell lysate protein was used for western blot analysis of STAR protein. (B) MA-10 cells were cultured in the medium with or without 25 μ M 22(R)hydroxylcholesterol (22R+ or 22R-) and then treated as described above. The medium was collected, and progesterone concentration in the medium was assessed by RIA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n = 3$; compared with the cells treated with 0.05 mM dbcAMP alone. *** $P < 0.001$; $n = 3$; compared with the cells treated with H89 or H8. In the presence of 22(R)hydroxylcholesterol in the cultures, there was no significant difference in progesterone production among the treatments.

Figure 6 Blocking Ca^{2+} influx through L-type Ca^{2+} channel depressed *Dax-1* gene expression in MA-10 mouse Leydig cells. MA-10 cells were cultured in serum-free Waymouth's medium with 15 μ M of the selective L-type Ca^{2+} channel blocker, verapamil, for 30 min, and then 0.05 mM dbcAMP was added to the cultures for 6 h. (A) The cells were collected, and 20 μ g cell lysate protein was used for western blot analysis of DAX-1 protein. (B and C) The cells were collected for total RNA isolation. *Dax-1* mRNA and *Star* mRNA were analyzed by RT-PCR with β -actin as an internal marker.

Dax-1 mRNA, which indicated that blocking L-type Ca^{2+} channel inhibited *Dax-1* gene transcription. When DAX-1 protein and mRNA were reduced, *Star* mRNA level increased dramatically (Fig. 6). The observations were further confirmed by quantitative real-time PCR analyses of *Dax-1* mRNA and *Star* mRNA, with *Dax-1* mRNA level being reduced and *Star* mRNA level being increased significantly by blocking L-type Ca^{2+} channels (Fig. 7).

Discussion

Previous studies suggested that Ca^{2+} influx through L-type Ca^{2+} channels may produce differential effects on *Star* gene expression in Leydig cells (Manna *et al.* 1999, Chen *et al.* 2007). The present study further indicates that Ca^{2+} influx through L-type Ca^{2+} channels is involved in an inhibitory effect on *Star* gene expression and regulates the sensitivity of Leydig cells to cAMP stimulation.

The inhibitory effect generated by Ca^{2+} influx through L-type Ca^{2+} channels on *Star* gene expression was demonstrated by three lines of evidence: 1) blocking Ca^{2+} influx through L-type Ca^{2+} channels with increasing levels of a selective blocker, nifedipine, induced a concentration-dependent increase in STAR protein expression in the cells

Figure 7 Real-time PCR analyses of *Dax-1* mRNA and *Star* mRNA in MA-10 mouse Leydig cells treated with verapamil. MA-10 cells were cultured in serum-free Waymouth's medium with 15 μ M of the selective L-type Ca^{2+} channel blocker, verapamil, for 30 min, and then 0.05 mM dbcAMP was added to the cultures for 6 h. The total RNA was isolated from the cells. The cDNA was synthesized from the RNA for real-time PCR analyses of *Dax-1* mRNA and *Star* mRNA, with β -actin as an internal marker. The levels of *Dax-1* mRNA and *Star* mRNA were adjusted by β -actin mRNA levels and expressed as fold of control. (A) For *Dax-1* mRNA: * $P < 0.05$; $n = 4$; compared with control. *** $P < 0.001$; $n = 4$; compared with the cells treated with 0.05 mM dbcAMP alone or verapamil alone. (B) For *Star* mRNA: *** $P < 0.001$; $n = 4$; compared with the cells treated with 0.05 mM dbcAMP alone or verapamil alone.

treated with 0.05 mM dbcAMP. Progesterone production by the cells was also increased, as STAR protein expression was enhanced by nifedipine; 2) the inhibitory effect generated by Ca^{2+} influx through L-type Ca^{2+} channels on STAR protein expression was confirmed by the experiments utilizing three different selective L-type Ca^{2+} channel blockers, verapamil, isradipine, and diltiazem respectively. Incubation of MA-10 cells with 25 μ M each of the blockers produced similar steroidogenic results, with each of them dramatically enhancing STAR protein expression and progesterone production; 3) the observations with nifedipine were corroborated by RT-PCR analyses of *Star* mRNA and also by the luciferase assays of *Star* promoter activity in the cells co-incubated with nifedipine and 0.05 mM dbcAMP, with both *Star* mRNA level and promoter activity being increased by the treatments. The results indicated that blocking L-type Ca^{2+} channels enhanced *Star* gene transcription. Similar inhibitory effect of Ca^{2+} influx through L-type Ca^{2+} channel on steroidogenesis was also reported in other types of steroidogenic cells (Barrett *et al.* 1991, 1995, Rossier *et al.* 1996).

The results from the present study further indicated that this inhibition on *Star* gene expression by Ca^{2+} influx through L-type Ca^{2+} channels was involved in regulating the sensitivity of Leydig cells to cAMP stimulation. Under normal circumstances, sub-threshold levels of cAMP (0.05 and 0.1 mM cAMP) are unable to induce STAR protein expression or a significant increase in progesterone production in MA-10 cells. However, in the presence of L-type Ca^{2+} channel blockers, the effectiveness of cAMP was dramatically increased, with 0.05 mM cAMP being able to induce a marked increase in STAR protein expression and also a significant increase in steroid hormone production. On the other hand, in the absence of these low levels of cAMP, blocking Ca^{2+} influx through L-type Ca^{2+} channels did not induce *Star* gene expression and steroidogenesis in MA-10 cells. We also observed that inhibition of PKA activity reversed the verapamil-increased STAR protein and progesterone production. These observations suggest that blocking L-type Ca^{2+} channels alone is unable to stimulate steroidogenesis. However, the blockers did attenuate the inhibitory effect and lowered the threshold of cAMP-stimulated STAR protein expression. Therefore, sub-threshold levels of cAMP are able to induce maximal levels of *Star* gene expression and steroid hormone production.

To understand how the Ca^{2+} influx through L-type Ca^{2+} channels generated the inhibitory effect on *Star* gene transcription and reduced the sensitivity of Leydig cells to cAMP stimulation, expression of DAX-1 protein, a transcriptional repressor of *Star* gene transcription, was examined. The results from western blot analyses showed that blocking Ca^{2+} influx through L-type Ca^{2+} channels reduced DAX-1 protein. This observation was confirmed by RT-PCR and real-time PCR analyses of *Dax-1* mRNA, with the level of *Dax-1* mRNA being reduced by blocking L-type Ca^{2+} channels in MA-10 cells cultured in the medium containing 0.05 mM cAMP, suggesting that Ca^{2+} influx through L-type Ca^{2+} channels acted at the transcriptional level of *Dax-1* gene expression. As shown in the present and previous studies (Wang *et al.* 2008), DAX-1 protein is constitutively expressed and maintained at a high level in MA-10 cells. DAX-1 generates a tonic inhibition on *Star* gene expression by binding to a hairpin structure in the *Star* promoter and depressing *Star* gene transcription (Zazopoulos *et al.* 1997). In addition, DAX-1 expression is able to reduce the level of steroidogenic factor-1 (SF-1) protein, an important transcriptional factor in *Star* gene expression, and also represses the transactivation potential of SF-1 on *Star* gene transcription (Manna *et al.* 2009). Thus, reduction in DAX-1 protein by blocking L-type Ca^{2+} channels attenuated the tonic inhibition of DAX-1 protein and increased the sensitivity of Leydig cells to cAMP stimulation.

These observations and previous studies indicated that Ca^{2+} influx through L-type Ca^{2+} channels not only produced positive effect (Manna *et al.* 1999), but also produced negative effect on *Star* gene expression. As described above, the negative effect resulted from the role

of Ca^{2+} influx through L-type Ca^{2+} channels in maintaining DAX-1 expression when cAMP levels were below the threshold of *Star* gene expression. This can be seen from the present study that blocking L-type Ca^{2+} channels using the blocker markedly reduced DAX-1 protein and increased *Star* gene expression. However, the steroidogenic effect of the L-type Ca^{2+} channel blocker is affected by the levels of cAMP. As concentrations of cAMP in the cultures increased to produce maximal level of STAR protein expression, cAMP stimulation reduces DAX-1 protein to the low levels (Jo & Stocco 2004, Manna *et al.* 2009), and also increases positive signals, including Ca^{2+} (Sullivan & Cooke 1986). Since DAX-1 protein was already reduced to such a low level, blocking L-type Ca^{2+} channel might not be able to effectively further reduce the inhibitory effect of DAX-1 protein, but reduced the positive effect of the Ca^{2+} influx through L-type Ca^{2+} channels on *Star* gene expression as previously reported (Manna *et al.* 1999).

In summary, the present study demonstrated that blocking L-type Ca^{2+} channels enhanced cAMP-induced *Star* gene transcription, STAR protein expression, and progesterone production in MA-10 mouse Leydig cells, indicating a role for Ca^{2+} influx through L-type Ca^{2+} channels in an inhibition of *Star* gene expression and steroidogenesis. This inhibitory effect may involve a role of Ca^{2+} influx through L-type Ca^{2+} channels in the regulation of *Dax-1* gene expression.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

The authors would like to acknowledge the support of NIH Grant AG025349 to XJW.

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Received in final form 5 October 2009

Accepted 12 October 2009

Made available online as an Accepted Preprint

12 October 2009