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Non-classical testosterone signaling is mediated by a G-protein-coupled receptor interacting with Gnα11

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ABSTRACT

Testosterone is known to mediate its effects by two different mechanisms of action. In the so-called "classical" pathway testosterone binds to cytosolic androgen receptors (AR), which essentially function as ligand-activated transcription factors. Once activated, these receptors bind to DNA and activate the expression of target genes. In the "non-classical" pathway, the steroid hormone binds to receptors associated with the plasma membrane and induces signaling cascades mediated through activation of Erk1/2. The precise nature of the membrane-associated AR, however, remains controversial. Although some assume that the membrane and cytosolic AR are identical, others propose that the AR of the membrane is a G-protein-coupled receptor (GPCR). To evaluate these two possibilities we first searched for testosterone-induced signaling cascades in the spermatogenic cell line GC-2. Testosterone was found to cause phosphorylation (activation) of Erk1/2, CREB, and ATF-1, consistent with its non-classical mechanism of action. Silencing of AR expression by means of siRNA did not influence testosterone-induced activation of Erk1/2, CREB, or ATF-1, indicating that this pathway is not activated by the classical cytosolic/nuclear AR. In contrast, when the expression of the G-protein Gnα11 is suppressed, the activation of these signaling molecules is abolished, suggesting that these responses are elicited through a membrane-bound GPCR. The results presented here and the identification of the testosterone-specific GPCR in future investigations will help to reveal and characterize new testosterone-mediated mechanisms associated not only with fertility and reproduction but perhaps also with other physiological processes.

1. Introduction

Steroid hormones influence the physiology of cells, organs and organisms in multiple ways. The classical view of their action proposes genomic effects as a result of their interactions with cytosolic steroid receptors (SR), which upon binding of the steroid dimerize, translocate into the nucleus, and modulate the expression of specific genes by acting as ligand-activated transcription factors [1,2]. A second, non-classical mode of steroid hormone action is characterized by rapid events that lead to the activation of cytosolic signaling cascades normally triggered by growth factors such as the Src/PI3K/Akt or the Src/Ras/Raf/Erk1/2 pathway [3,4]. These signaling events originate at the surface of plasma membranes, where specific steroid receptors localized within rafts mediate the rapid activation of intracellular signaling cascades [5]. These membrane-bound steroid receptors are often G-protein coupled receptors (GPCR) and therefore different from the nuclear SR [6–8].

Testosterone undoubtedly triggers both classical and non-classical pathways of action, but the nature of the receptor involved in these actions is a source of controversy. While some investigators favor the exclusive participation of the well-characterized cytosolic/nuclear androgen receptor (AR) in both pathways [9], others propose a membrane-bound AR, possibly from the family of G-protein-coupled receptors (GPCR), as mediator of several testosterone-induced effects [10–14].

Testosterone action on cells of the male reproductive system is essential for spermatogenesis and the maturation of spermatogonia to spermatozoa. CREB activation in Sertoli cells, which is required for the survival of spermatocytes and the production of mature spermatozoa [15], is triggered by testosterone interactions with the AR via the activation of the c-Src/c-Raf/Erk1/2 signaling cascade, part of the non-classical testosterone signaling pathway [9,16,17]. The processes of spermatogenesis and the maturation of spermatogonia to spermatozoa also depend on the activation of Erk1/2 and other mitogen-activated protein kinases (MAPK) [18,19]. In addition, Erk1/2 activation is an absolute requirement for the production of haploid spermatozoa [20,21].

The question still to be answered, however, is whether all of these effects are due solely to the interaction of testosterone with the classical AR localized in Sertoli cells or whether testosterone might exert some of its actions on other cells of the reproductive system by interacting with a different, thus-far unidentified receptor. Should the latter possibility be the case, one would have to supplement or even revise some of the knowledge concerning the importance of testosterone for male

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reproduction. Having this in mind we addressed the role of the classical AR in testosterone-induced signaling in the spermatogenic cell line GC-2. The results show that in addition to the cytosolic/nuclear AR, there is also a GPCR that mediates the non-classical testosterone pathway in the GC-2 cells. The findings indicate that testosterone may initiate some of its actions by detouring the classical AR of Sertoli cells and interacting more directly with GPCR of the other cells of the male reproductive system.

2. Materials and methods

2.1. Cell culture

The spermatogenic cell line GC-2 spd (ts) [22] (hereafter referred to as GC-2) was cultured in DMEM (1×) high glucose containing 1% L-glutamine (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin combination (100 U/ml of each). Cells were incubated in a humidified incubator at 31 °C under 5% CO_2. The medium was renewed every two days. Experiments were carried out after the 20th day of culture (third passage).

2.2. Cell lysates

GC-2 cells were seeded at a density of 10^5 cells in 5-cm culture dishes and grown as described above until they reached 70–80% confluence. Cells were then incubated for 24 h with 1% FCS before testosterone dissolved in ethanol was added to the medium to reach a final concentration of 1 nM (see Supplementary data regarding choice of concentration). Controls received the equivalent amount of ethanol. After 30 min of incubation (see Supplementary data regarding choice of incubation time) the medium was removed by aspiration and cells were washed twice with ice-cold phosphate-buffered saline (PBS; without Ca^{2+} or Mg^{2+}; Gibco) and lysed in 400 μl of a commercially available cell lysis buffer (Cell Signaling Technology, Frankfurt, Germany) according to the protocol of the provider. Immediately before use, 1 μM PMSF, 1× protease inhibitor cocktail (Roche, Mannheim, Germany), and 2 μg/ml pepstatin were added to the lysis buffer. All lysis steps were carried out on ice. After 10 min of incubation cells were harvested with a scraper, transferred into vials, and sonicated 5 times for 5 s with intervals of 2 s. The reaction vials were then centrifuged at 13,000 ×g for 10 min at 4 °C. The protein content of the supernatants was determined at 540 nm using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Southfield, MI, USA) and a Labsystems (Helsinki, Finland) plate reader. The lysis buffer was included in the bovine serum albumin protein standard. Aliquots of the supernatant taken for further analysis were stored at −20 °C.

2.3. SDS-PAGE and western blotting

A total of 8 μg protein from cell lysates was separated by SDS-PAGE on slab gels containing 10% acrylamide and 0.3% N,N′-methylene-bis-acrylamide. Biotinylated proteins (Cell Signaling Technology, Frankfurt, Germany) served as molecular weight markers. After electrophoresis proteins were blotted onto PVDF membranes (Millipore, Bedford, MA, USA) for 30 min at 200 mA. Specific protein bands were visualized by incubating the membranes with primary antibodies according to the protocol of the providers (Table 1) and the appropriate secondary antibody of the enhanced chemiluminescence kit (ECL; Pierce). For the simultaneous detection of p-CREB and p-ATF-1, western blots were probed with an antibody that cross-reacts specifically with the two phosphorylated proteins (Cell Signaling Technology). Horseradish peroxidase-conjugated anti-biotin IgG (Cell Signaling Technology) at a dilution of 1:2000 was included in the mixture containing the secondary antibody in order to detect the biotinylated molecular weight marker. The resulting chemiluminescence was recorded by exposure to film, which was analyzed by the TotalLab gel image analysis software (biostep, Jahnsdorf, Germany).

2.4. RT-PCR for the detection of mRNA/cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), androgen receptor (AR), and guanine nucleotide binding protein, alpha 11 (Gnα11)

Total mRNA was isolated from GC-2 cells by following the protocol of the provider of the SV Total RNA Isolation System (Promega, Mannheim, Germany). Reverse transcription and PCR amplification of mRNA/cDNA of interest were carried out by following the protocol of the Reverse Transcription System provider (Promega). For PCR amplification a total of 10 ng/μl of cDNA was incubated with 20 pmol/ml of each primer, 10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dNTPs, and 2 units *Taq* DNA polymerase. The final volume of the solutions was 25 μl. PCR was carried out in a MasterCycler Gradient (Eppendorf, Hamburg, Germany). Samples were incubated at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at a temperature of 54 °C for 1 min, and cDNA extension at 72 °C for 45 s. After amplification, a final extension at 72 °C was performed for 10 min.

GAPDH-specific mRNA/cDNA was detected using the oligonucleotide 5′GGAGATTGTTGCCATCAACG3′ as forward primer and 5′CACAATGCCA AAGTTGTCA3′ as reverse primer. These primers amplify a fragment of 430 bp between bases 128 and 557 of mouse GAPDH-specific mRNA.

AR-specific mRNA/cDNA was amplified under the same conditions used for the amplification of GAPDH. Forward and reverse primers were the oligonucleotides 5′AGCGCAATGCCGCTATGGGG3′ and 5′GTGG GGCTGCCAGCATTGGA3′, respectively. These amplify a 708-bp fragment of mouse AR-specific mRNA localized between bases 1220 and 1927.

Gnα11-specific mRNA/cDNA was amplified under the same conditions as GAPDH. Forward and reverse primers were the oligonucleotides 5′GAACCGGGAAGAGGTAGGG3′ and 5′GACCAAGTGTGAGTGCAGGA3′, respectively. These amplify a 917-bp fragment of mouse Gnα11-specific mRNA localized between bases 70 and 986.

2.5. Silencing androgen receptor expression via siRNA

Expression of the androgen receptor was silenced by using commercially available siRNA and by following the protocol of the provider (Stealth™ RNAi; Invitrogen, Karlsruhe, Germany). The oligonucleotide pair used was: 5′CCAGAUUCCUUUGCUGCCUUGUUAU3′ and AUAACA AGGCAGCAAAGGAAUCUGG3′ (AR-siRNA). Control cells were treated with Stealth™ RNAi Negative Control, provided in the kit. Transfection efficiency was estimated by the Block-iT™ Transfection Kit (Invitrogen,

Table 1
Antisera used and their providers (IF = immunofluorescence; WB = western blot).

Antibody	Catalog no.	Provider	Address
Anti-AR (H-280) (for IF)	sc-13062	Santa Cruz Biotechnology, Inc.	Heidelberg, Germany
Anti-phospho-CREB and anti-phospho-ATF-1 (for WB)	4276	Cell Signaling Technology	Frankfurt am Main, Germany
Anti-phospho-CREB (for IF)	9198	Cell Signaling Technology	Frankfurt am Main, Germany
Anti-phospho-ATF-1 (for IF)	2456-1	Epitomics	Burlingame, USA
Anti-phosho-Erk1/2 (for WB and IF)	4370	Cell Signaling Technology	Frankfurt am Main, Germany
Anti-total Erk1/2 (for WB)	9102	Cell Signaling Technology	Frankfurt am Main, Germany
Anti-pan-Actin (for WB)	4968	Cell Signaling Technology	Frankfurt am Main, Germany

Karlsruhe, Germany) according the protocol of the provider. After incubation of the GC-2 cells for 72 h with the various siRNA primer pairs or the negative control siRNA, mRNA for RT-PCR was isolated as described above (previous paragraph). A second set of cells was stimulated with 1 nM testosterone and used for the detection of activated Erk1/2, CREB, and ATF-1 by immunofluorescence, as described further below. Finally, a third set of cells was stimulated with 1 nM testosterone and used for the isolation of cell lysates to be investigated in western blots.

2.6. Silencing the expression of Gnα11 via siRNA

Control GC-2 cells were treated with the siRNA Negative Control as supplied by the provider (Silencer® Select siRNA; Invitrogen). For silencing Gnα11 expression, cells were treated like control cells with the exception that commercially available siRNA directed against the expression of Gnα11 (Silencer® Select siRNA; Invitrogen) was used. The oligonucleotide pair used was 5′CAAGAUCCUCUACAAGUAUTT3′ and 5′AUACUUGUAGAGGAUCUUGAG3′ (Gnα11-siRNA). All other steps were the same as described in the previous paragraph.

2.7. Immunofluorescence

GC-2 cells that had been treated with siRNA to silence either AR or Gnα11 were incubated with vehicle alone or vehicle plus 1 nM testosterone for 30 min. The medium was then aspirated and the cells were fixed using 200 μl of ice-cold methanol containing a total of 20 ng of DAPI (4′,6-diamidino-2-phenylindole). After 15 min of incubation at RT, the DAPI solution was aspirated and slides were allowed to dry for 15 min before washing 3 times with 500 μl PBS. The cells were then blocked with 10% FCS and 0.3% Triton-X100 in PBS for 1 h at RT. The first antibody (Table 1), diluted as recommended by the provider, was then added and incubation was continued for 1 day at 4 °C in a humidified chamber. The antibody against p-Erk1/2 was from Cell Signaling Technology. The antibody against p-ATF-1 was from Epitomics (Burlingame, CA, USA). This antibody is p-ATF-1 specific and does not interact with p-CREB. For the specific detection of p-CREB, an antibody from Cell Signaling Technology was used with negligible interaction with p-ATF-1. The antibody against the androgen receptor was from Santa Cruz Biotechnology (Heidelberg, Germany).

The slides were then washed 3 times for 5 min each with 500 μl PBS. Staining was achieved by incubating for 20 min at room temperature with an Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) diluted at 1:500 in 2% FCS, 0.1 Triton X100 in PBS. Images were obtained by an inverse Olympus IX81 microscope equipped with the corresponding fluorescence system (Olympus, Hamburg, Germany). Fluorescence within cells was measured by using the software program ImageJ (freely available at http://rsbweb.nih.gov/ij/). A total of 30 cells within or closest to the diagonals of the square optical field were considered. Data points were transferred to and analyzed by the software program GraphPad Prism4 (GraphPad Software, Inc., La Jolla, CA, USA).

2.8. Statistical analysis

Loading differences in the various western blots were corrected by taking into consideration the optical density of unphosphorylated Erk1/2 bands or total actin, detected in western blots that were run in parallel. Data were analyzed by GraphPad Prism4 software and by applying one-way ANOVA with repeated measures and Dunnett's comparison of all data to the control. Significance was accepted at $p < 0.05$.

3. Results

3.1. Silencing the androgen receptor by siRNA

After 72 h of incubation of cells with the siRNA oligonucleotides against the AR, mRNA was isolated for RT-PCR. Fig. 1 shows an agarose gel with the RT-PCR products obtained before and after treatment of the GC-2 cells with siRNA to silence AR expression. While having no effect on the expression of GAPDH-specific mRNA/cDNA, AR-siRNA reduced the biosynthesis of AR-specific mRNA/cDNA to a great extent (Fig. 1). The expression of GAPDH- or AR-specific mRNA was not affected by negative control siRNA (nc-siRNA; Fig. 1). Nevertheless, since a small amount of AR-specific mRNA/cDNA was also detected after treatment of the cells with AR-siRNA, and because silencing of mRNA might not necessarily lead to a rapid decrease in the expression of the targeted protein, we addressed by immunofluorescence whether the AR protein

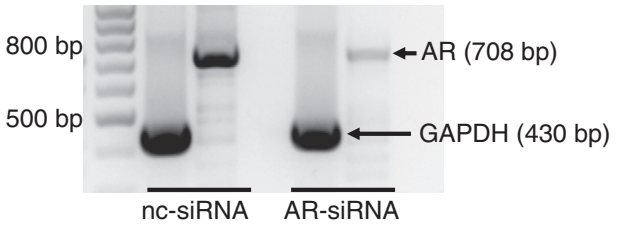

Fig. 1. Silencing expression of AR-specific mRNA using siRNA. GC-2 cells were treated with either AR-siRNA or negative-control siRNA (nc-siRNA). Total RNA was then isolated and subjected to RT-PCR to amplify AR-specific mRNA/cDNA fragments of 708 bp. Treatment of the cells with AR-siRNA abolished the expression of AR-specific mRNA/cDNA. The amount of GAPDH mRNA was not affected by either nc-siRNA or AR-siRNA, indicating specific silencing of AR mRNA expression by AR-siRNA.

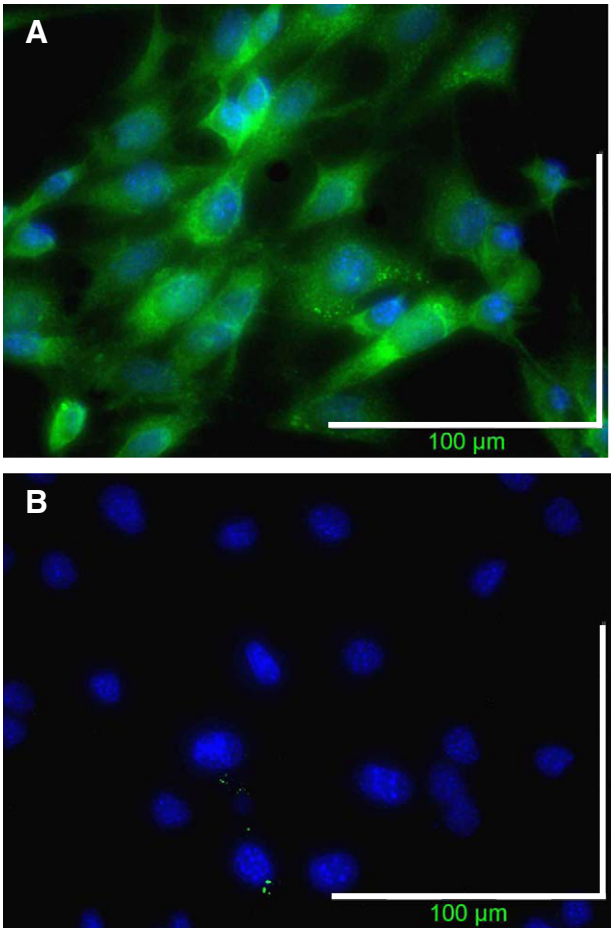

Fig. 2. Silencing AR protein expression by siRNA. Cells treated as described in Fig. 1 were fixed in methanol and incubated with a primary antibody against the AR and a fluorescent secondary antibody (rabbit anti-goat IgG-FITC green). Nuclei were stained with DAPI. (A) All cells treated with nc-siRNA show green fluorescence, indicating the presence of the AR. (B) When cells were treated with AR-siRNA, no AR protein was detected by the combination of the antibodies used in A.

was still present in the cells despite the reduction of AR-specific mRNA/cDNA by siRNA. Although green fluorescence, indicating the expression of the AR protein, was visible in every GC-2 cell in the image shown in Fig. 2A, it was entirely missing after treatment of the cells with AR-siRNA to prevent expression of AR-specific mRNA (Fig. 2B). It is therefore likely that the weak AR-specific signal seen after treatment

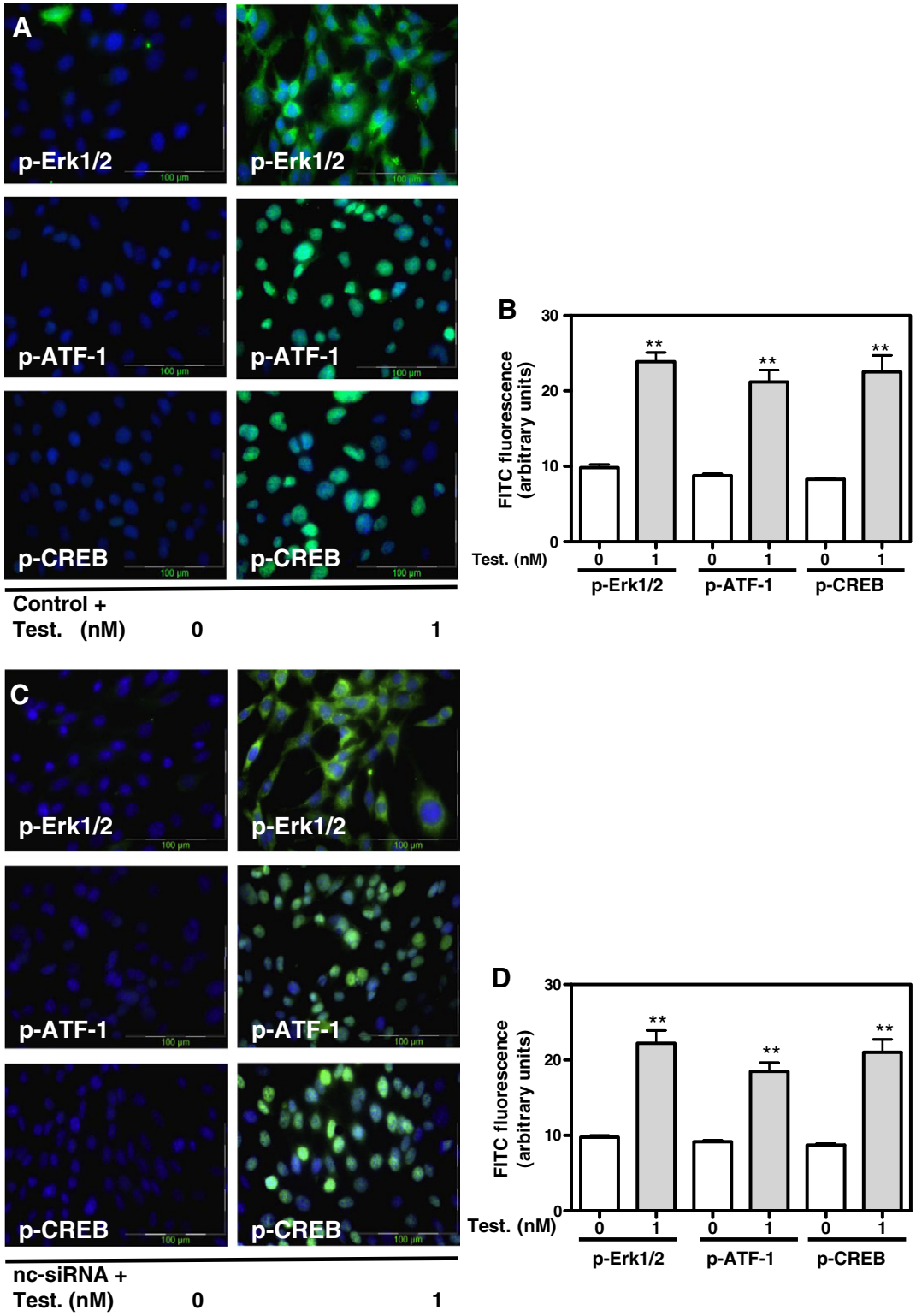

Fig. 3. Testosterone-induced activation of Erk1/2, ATF-1, and CREB in the presence or absence of AR detected by immunofluorescence. (A) GC2 cells that had not been treated with siRNA were incubated with testosterone and then fixed in methanol; nuclei were stained with DAPI. p-Erk1/2, p-ATF-1, or p-CREB was identified by using specific primary antibodies (Table 1) and an Alexa Fluor 488-labeled secondary antibody. Treatment of cells with 1 nM testosterone for 30 min triggered formation of p-Erk1/2, p-ATF-1 and p-CREB visualized as green staining (right panels). (B) Analysis of data like that shown in A; n = 30; means ± SEM; ** = $p \leq 0.01$. (C) Cells were treated with nc-siRNA before exposure to testosterone as in A; this did not affect the testosterone-induced activation of Erk1/2, ATF-1, or CREB. (D) Activation by testosterone was significant, as in A and B; n = 30; means ± SEM; ** = $p \leq 0.01$. (E) Cells were treated with AR-siRNA to silence AR expression. Fluorescence signals indicating activation of Erk1/2, CREB, or ATF-1 in response to testosterone were not affected by the silencing of AR expression. (F) Activation by testosterone was significant, as in A–D; n = 30; means ± SEM; ** = $p \leq 0.01$.

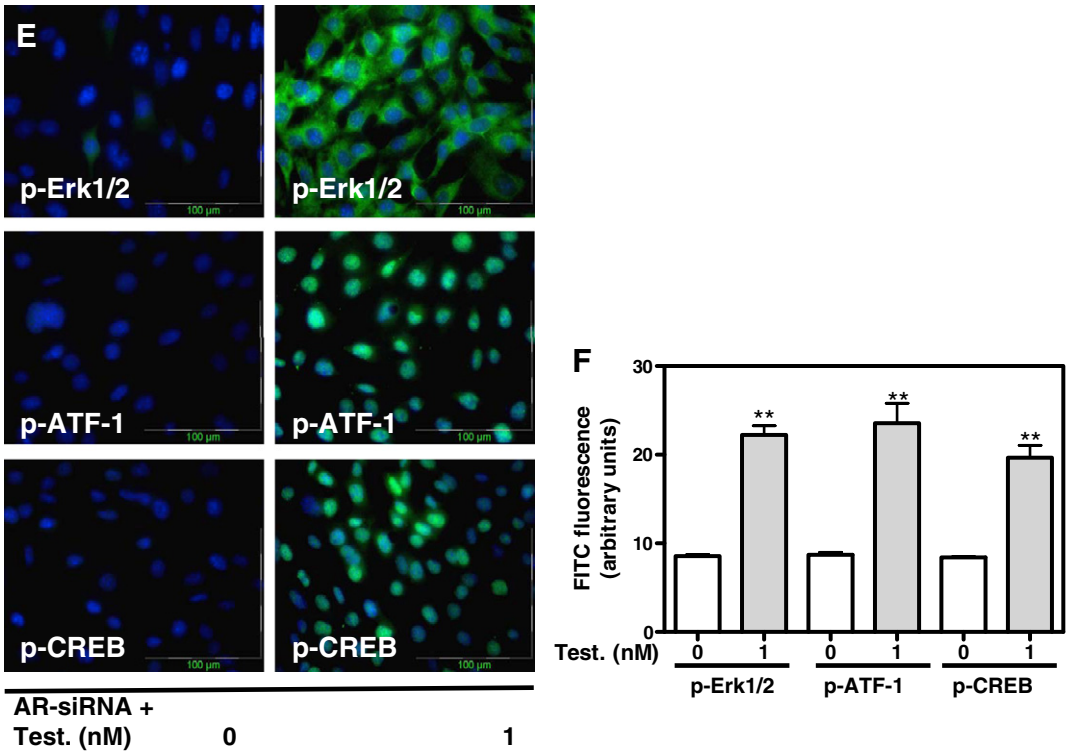

Fig. 3 (*continued*).

with AR-siRNA (Fig. 1) is the result of the strong amplification effects of the RT-PCR.

3.2. Testosterone-induced activation of Erk1/2, CREB, and ATF-1 in GC-2 cells in the presence or absence of AR

In the non-classical action of testosterone, the steroid hormone triggers the Src/Ras/Raf/Erk1/2 signaling cascade that results in the activation of the transcription factor CREB. Thus, our first aim was to examine whether testosterone activates elements of this signaling cascade in the spermatogenic GC-2 cells. In this respect we addressed a possible testosterone-induced activation of Erk1/2, CREB, and ATF-1. Both ATF-1 and CREB are members of the bZIP superfamily of transcription factors and stimulate transcription when activated by phosphorylation at either Ser63 (ATF-1) or Ser133 (CREB). Simultaneous activation of the two related transcription factors has been shown previously [23], and we investigated whether testosterone might act on GC-2 cells in a similar way.

Cells were incubated with either 0 or 1 nM testosterone for 30 min and then subjected to a fixation/immunostaining procedure as described under "Materials and methods". Phosphorylated forms of Erk1/2, ATF-1, or CREB were detected by using appropriate antibodies (Table 1). Fig. 3A demonstrates that testosterone triggered activation of the kinase and of both transcription factors in a highly significant way (Fig. 3B). Erk1/2 activation was seen in the form of green fluorescence spread over the entire area of the testosterone-treated cells, while the transcription factors ATF-1 and CREB (Fig. 3A) were visible as green fluorescent signals within the nucleus. To our knowledge this is the first report demonstrating ATF-1 activation by testosterone.

Remarkably, comparable results were obtained with cells that were treated with AR-siRNA to silence AR expression. Testosterone induced a clear activation of Erk1/2, ATF-1 and CREB that was not affected by the absence of AR (Fig. 3E, F). Treatment with negative-control siRNA (nc-siRNA) did not affect the testosterone-induced stimulation of Erk1/2, ATF-1, or CREB (Fig. 3C, D).

3.3. Detection of p-Erk1/2, p-ATF-1, and p-CREB in western blots in the presence or absence of AR

Since immunofluorescence only reliably stains cells or proteins residing within the optical field of the microscope, we carried out western blot experiments to obtain a representative average by measuring the testosterone action on all cells of the incubation mixture. Testosterone effects on GC-2 cells treated with nc-siRNA were compared to its effects on cells treated with siRNA to silence AR expression (AR-siRNA). As can be seen in Fig. 5B, treatment of GC-2 cells with AR-siRNA did not impair the ability of testosterone to induce activation of Erk1/2 (Fig. 4C), which is consistent with the results shown in Fig. 3. The total amount of Erk1/2 was not affected by the steroid hormone (Fig. 4A).

Similarly, in the absence of AR testosterone still caused activation of ATF-1 and CREB. In western blots with an antibody that cross-reacts with p-CREB and p-ATF-1 (Fig. 5A), we observed significant activation of both transcription factors following 30 min of incubation with 1 nM testosterone (Fig. 5B and C). These results, which are consistent with those shown in Fig. 3, indicate that the non-classical signaling pathway of testosterone is not triggered by the interaction of the steroid with the known cytosolic/nuclear AR.

3.4. Testosterone-induced activation of Erk1/2, CREB, and ATF-1 in GC-2 cells in the presence or absence of Gnα11

Many hormones, among them steroid hormones, elicit their actions through G-protein-coupled receptors (GPCRs) [24–27]. In a previous investigation we found that dehydroepiandrosterone sulfate (DHEAS) induces signaling cascades in GC-2 cells that overlap with the non-classical pathway of testosterone; this signaling cascade is mediated through a GPCR that interacts with Gnα11 [28]. For that reason, we investigated a possible involvement of GPCRs in the testosterone-induced signaling cascade by silencing Gnα11 expression in these cells.

The results from RT-PCR shown in Fig. 6 demonstrate that after treating GC-2 cells with the Gnα11-siRNA, the expression of Gnα11-

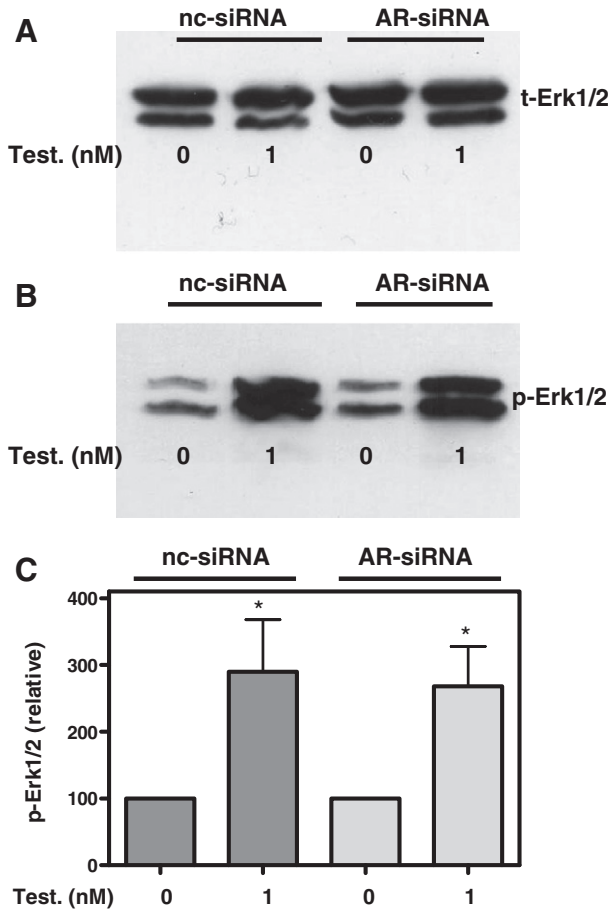

Fig. 4. Western blot analysis of p-Erk1/2 after silencing AR expression by siRNA. Cells were treated with either nc-siRNA or with AR-siRNA. After 30 min of incubation in the presence or absence of 1 nM testosterone, cell lysates were prepared and probed in western blots. (A) Incubation with 1 nM testosterone had no effect on total Erk1/2. (B) Testosterone stimulated the formation of p-Erk1/2 independent of whether cells were treated with nc-siRNA or AR-siRNA. (C) The data in the bar graph were corrected for the amount of total Erk1/2 as shown in A ($n = 3$; means ± SEM; * = $p \leq 0.05$).

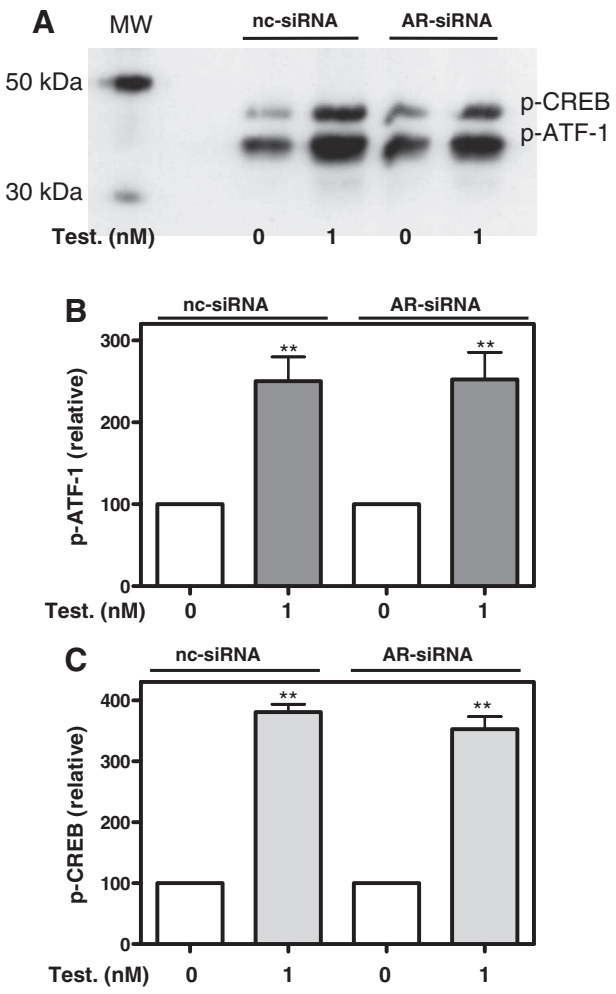

Fig. 5. Western blot analysis of p-ATF-1 and p-CREB after silencing AR expression by siRNA. Conditions are the same as in Fig. 4. (A) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA or AR-siRNA to the same extent. Analysis of pooled data like those shown in A ($n = 3$; means ± SEM; ** = $p \leq 0.01$). The data in the bar graph were corrected for the amount of total Erk1/2 as shown in Fig. 4A, which was used as a gel loading control.

specific mRNA/cDNA was considerably reduced. Untreated GC-2 cells and cells treated with either nc-siRNA or with siRNA against Gnα11 (Gnα11-siRNA) were incubated with 0 or 1 nM testosterone for 30 min. The images in the right-hand panels of Fig. 7A show the stimulation (phosphorylation) of Erk1/2, ATF-1, and CREB by 1 nM testosterone in GC-2 cells that had not been treated with any kind of siRNA. These data are consistent with the results shown in Fig. 3A, and here, too, the total cell-associated fluorescence corresponding to active Erk1/2, ATF-1, or CREB was significantly higher in cells exposed to testosterone than the fluorescence measured in the absence of the steroid (Fig. 7B). Similar results were obtained when cells were treated with nc-siRNA (Fig. 7C and D). When cells were treated with Gnα11-siRNA, exposure to testosterone had no effect (Fig. 7E, right-hand panels), clearly demonstrating the involvement of Gnα11 in mediating the testosterone-induced signaling that leads to Erk1/2, ATF-1, or CREB activation. Fluorescence corresponding to active Erk1/2, ATF-1, or CREB after 30 min of incubation with 1 nM testosterone was negligible, corresponding roughly to the fluorescence measured in the absence of the steroid (Fig. 7F).

3.5. Detection of p-Erk1/2, p-ATF-1, and p-CREB in western blots in the presence or absence of Gnα11

The western blot shown in Fig. 8 confirms the immunofluorescence experiments shown in Fig. 7. Silencing Gnα11 expression by

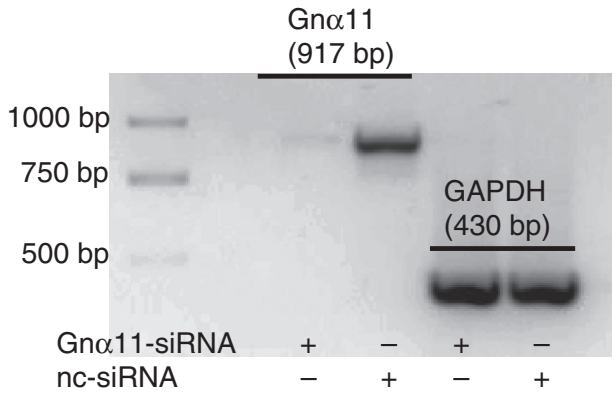

Fig. 6. Silencing expression of Gnα11-specific mRNA/cDNA by siRNA. Cells were incubated with nc-siRNA or Gnα11-siRNA to silence Gnα11-mRNA expression. Total RNA was then isolated and subjected to RT-PCR to amplify Gnα11-specific mRNA/cDNA fragments of 917 bp. While Gnα11-specific mRNA/cDNA was clearly present in cells treated with nc-siRNA, treatment of the cells with Gnα11-siRNA greatly reduced the expression of Gnα11-specific mRNA/cDNA. GAPDH-specific mRNA/cDNA expression was the same in cells treated with either nc-siRNA or Gnα11-siRNA.

transforming GC-2 cell Gnα11-siRNA (Fig. 8B) led to abolition of the testosterone-induced activation of Erk1/2 (Fig. 8B: p-Erk1/2). At the same time, cells treated with nc-siRNA still responded to testosterone with Erk1/2 activation (Fig. 8B and C). The expression of total Erk1/2 was not influenced by treatment with either nc-siRNA or Gnα11-siRNA (Fig. 8A).

Treatment of GC-2 cells with Gnα11-siRNA also prevented testosterone-induced activation of ATF-1 and CREB (Fig. 9A), while treatment with nc-siRNA did not impair significant activation of the two transcription factors (Fig. 9A, C, and D). The detection of total actin in the lysates served as loading control. Neither of the two siRNAs nor testosterone influenced its expression (Fig. 9B).

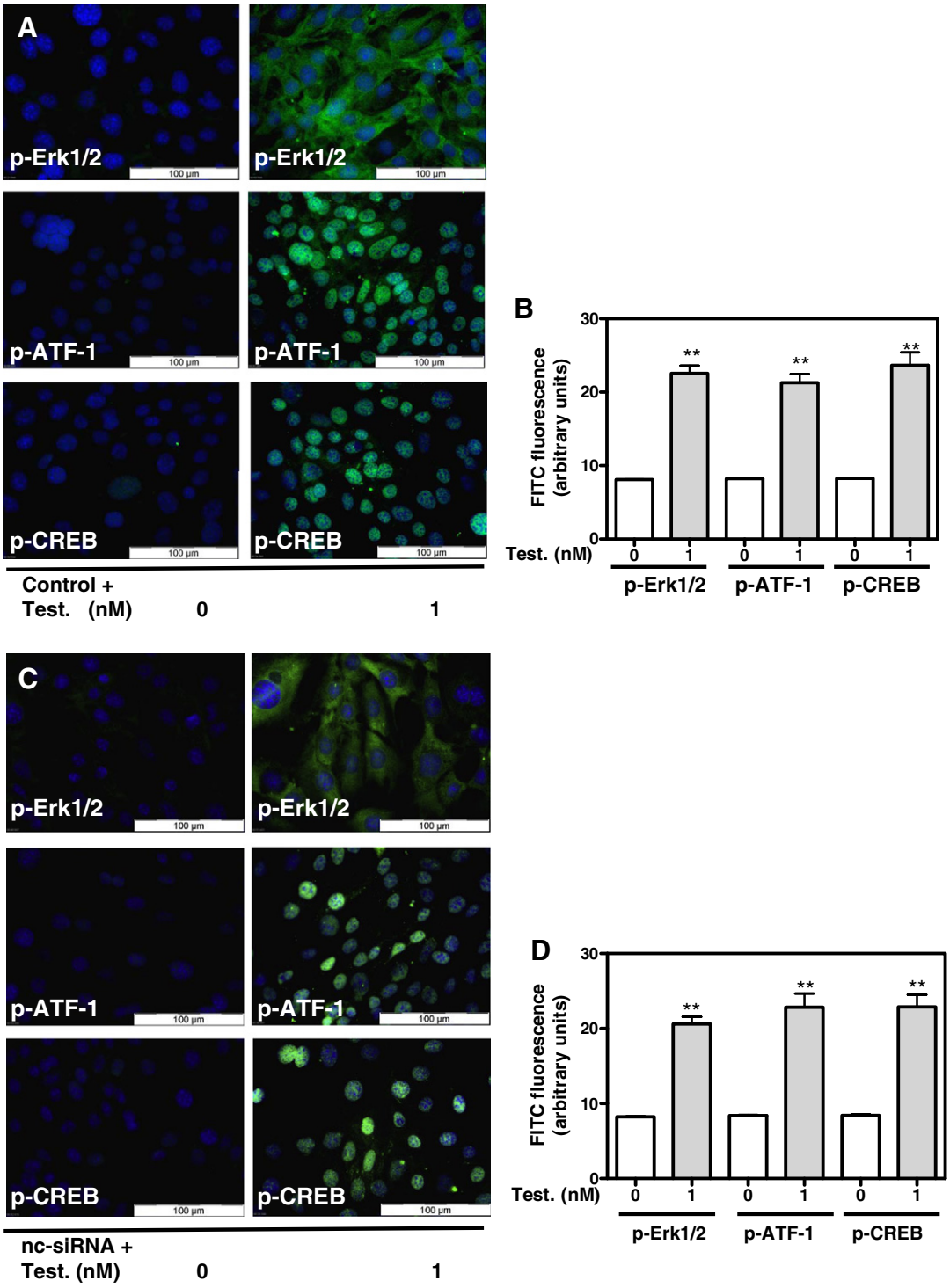

Fig. 7. Detection of testosterone-activated Erk1/2, ATF-1, and CREB by immunofluorescence after silencing Gnα11 expression by siRNA. (A) After treatment with 1 nM testosterone (right panels) almost every single cell in the optical field was fluorescent, indicating activation of Erk1/2, ATF-1, and CREB (top to bottom). (B) In the presence of testosterone the activation is significantly higher than in the untreated controls. (C) When cells were treated with negative control siRNA (nc-siRNA), they respond to testosterone like cells that had not been treated with any kind of siRNA, as shown in (A). (D) Here, too, testosterone induces a highly significant activation of Erk1/2, ATF-1 and CREB. (E) After silencing Gnα11 expression by Gnα11-siRNA testosterone fails to stimulate Erk1/2 and either of the transcription factors ATF-1 and CREB (right panels, top to bottom). (F) Statistically there is no difference in p-Erk1/2, p-ATF-1 and p-CREB in cells that were exposed to testosterone and untreated cells (for all statistical data shown in B, D and F: $n = 30$; means ± SEM; ** = $p \leq 0.01$).

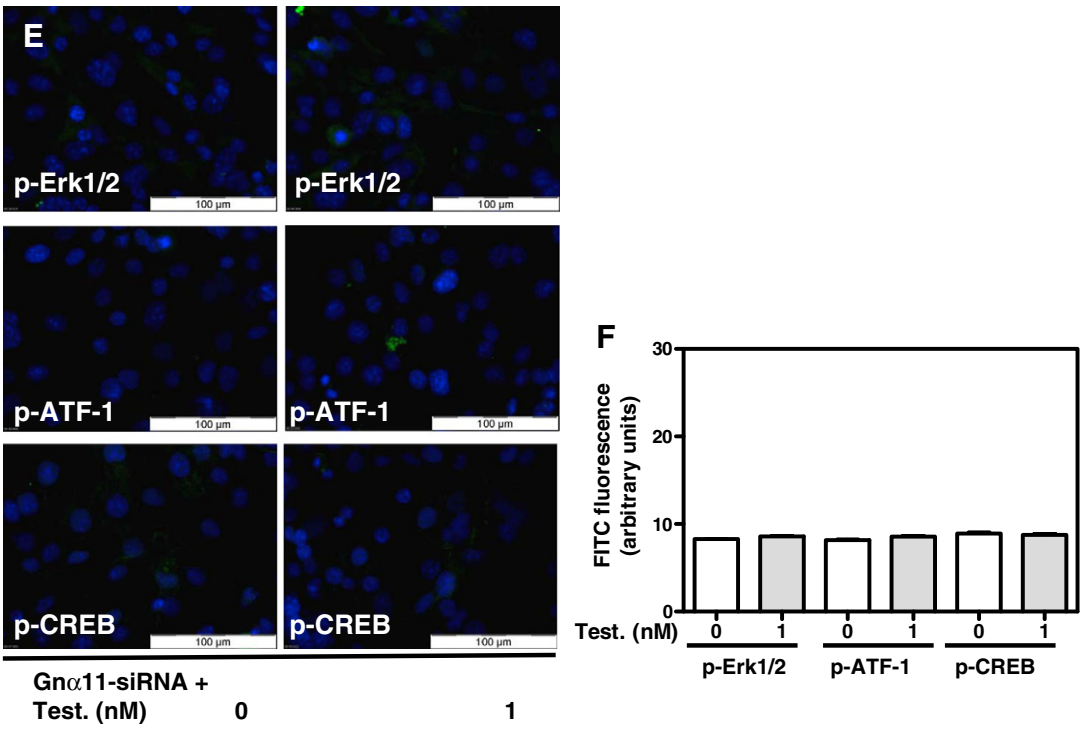

Fig. 7 (*continued*).

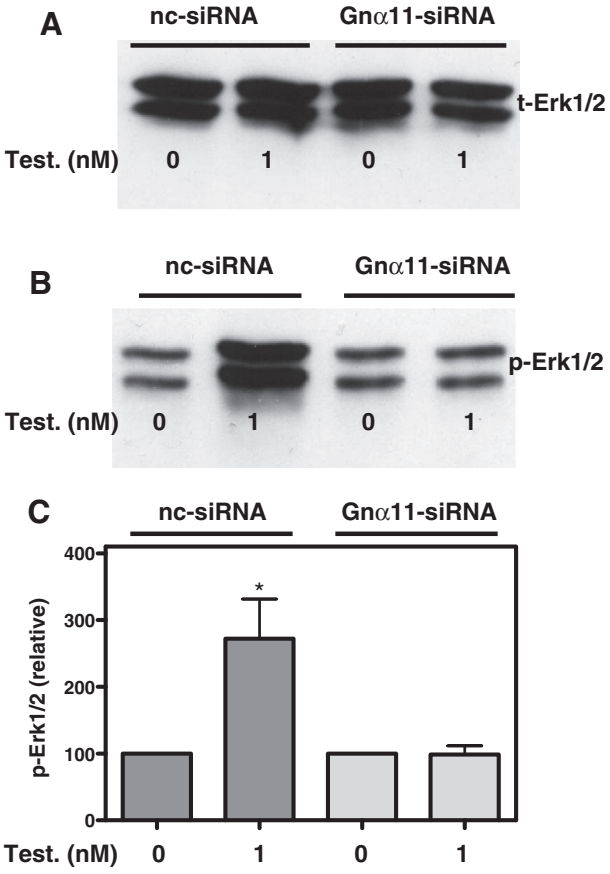

Fig. 8. Western blot analysis of p-Erk1/2 after silencing Gnα11 expression by siRNA. GC-2 cells were treated with nc-siRNA or Gnα11-siRNA followed by 0 or 1 nM testosterone as in Fig. 7. Cell lysates were probed for p-Erk1/2 as in Fig. 4. (A) Total Erk1/2 was not affected by testosterone or Gnα11-siRNA incubation. (B) Testosterone stimulated Erk1/2 activation in cells treated with nc-siRNA but had no effect in cells that had been treated with Gnα11-siRNA. (C) For statistical analysis, data were corrected for the amount of total Erk1/2 as shown in A ($n = 3$; means ± SEM; * = $p \leq 0.05$).

4. Discussion

Testosterone affects the physiology of various tissues by triggering multiple signaling pathways. In the classical view of its action the steroid diffuses into the cell, binds to a cytosolic AR that is associated with Hsp90 and Hsp70 and inactive, and induces the release of both Hsp; the AR then undergoes dimerization and translocates as a dimer into the nucleus. By acting as a transcription factor, the AR/steroid complex induces genomic responses that lead to the expression of specific genes [1,2].

In the non-classical pathway the steroid hormone binds to membrane-associated receptors and induces activation of various kinases, leading to a great spectrum of cellular responses [9,29,30]. The AR mediating these types of signaling cascades has not yet been identified. In muscle cells testosterone effects leading to Erk1/2 activation, cytosolic [Ca^{2+}] elevation, and protein kinase C activation seem to be mediated by its interactions with GPCR [13,14]. Similar effects of testosterone on [Ca^{2+}] are seen in Sertoli cells, where the phospholipase C inhibitor U73122 or pertussis toxin prevent these testosterone actions, thus indicating the involvement of GPCR [31]. A second non-classical signaling pathway of testosterone in Sertoli cells leads to the activation of the Ras/Raf/Erk1/2/CREB cascade [9,32]. Experiments utilizing siRNA to silence expression of the cytosolic/nuclear AR have provided evidence for its involvement in the mediation of the signaling cascade leading to CREB activation [33]. It is thought that some of the AR temporarily associate with the plasma membrane of Sertoli cells, and by interacting with testosterone, they induce stimulation of c-Src followed by the activation of epidermal growth factor receptor and the other members of the signaling cascade [9,32]. It is not known whether dimerization of AR is required for this cascade or what happens to Hsp70 and Hsp90.

The actions of testosterone on Sertoli cells are essential for the maturation of male germ cells into spermatozoa [17,34]. Nevertheless, AR are not localized solely in Sertoli cells; they are also found in Leydig cells and peritubular myocytes [35–39]. The presence of classical AR in germ cells is controversial: whereas several publications challenge its expression in germ cells in toto [38,40], others identify AR in human sperm [41], in sperm of the Bonnet monkey [42], or the midpiece of

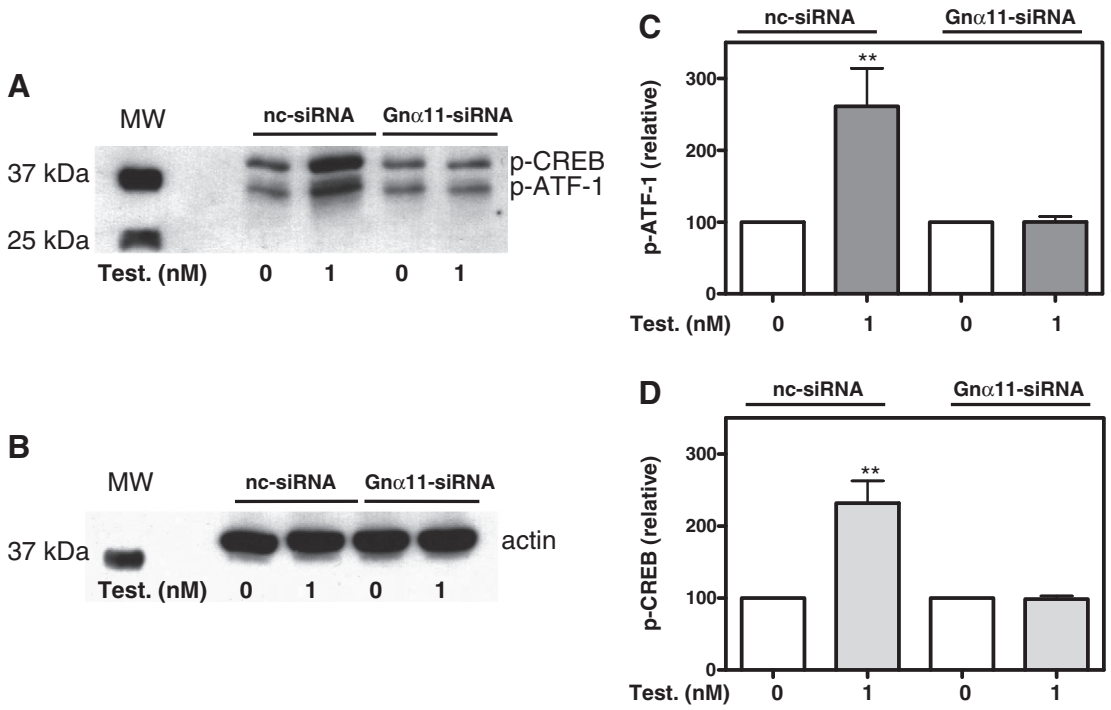

Fig. 9. Western blot analysis of p-ATF-1 and p-CREB after silencing Gnα11 expression by siRNA. Lysates were isolated after treatment of the cells with either nc-siRNA or Gnα11-siRNA. (A) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA but not in cells that had received Gnα11-siRNA. (B) Expression of total actin was not affected by the treatment of the cells with either type of siRNA or testosterone. (C and D) For statistical analysis data were corrected for the amount of total actin as shown in B ($n = 3$; means ± SEM; ** = $p \leq 0.01$).

flagella of mature human sperm [43]. In contrast to these contradicting reports, the presence of AR in spermatogonia seems to be generally accepted [37,39,44,45], suggesting a direct role of testosterone in the early stages of spermatogenesis. For this reason, and because testosterone might act on other cells of the gonad through GPCR and influence their physiology, we investigated non-classical testosterone-induced signaling in the spermatogenic cell line GC-2 that was shown previously to express AR [28].

GC-2 cells respond to testosterone with activation (phosphorylation) of Erk1/2 and the transcription factors CREB and ATF-1 (Figs. 3–5). This overlap with the non-classical action of testosterone [9] suggested that, like in Sertoli cells, classical AR are also involved in propagation of testosterone-induced signaling in the spermatogenic GC-2 cells. This possibility was addressed in a series of experiments after restricting AR expression at the mRNA (Fig. 1) and protein level (Fig. 2) by means of siRNA. The results summarized in Figs. 3–5 clearly show that silencing of classical AR does not affect the induction of testosterone-induced signaling in GC-2 cells.

These data demonstrate that AR do not participate in the non-classical testosterone signaling identified in GC-2 cells; nevertheless, they contrast with earlier studies also employing AR-specific siRNA that implicated a role of classical AR in Erk1/2 and CREB activation in Sertoli cells [33]. In the absence of any alternative and satisfactory way to explain the discrepancy between the two investigations, one can speculate at the current stage that the differences arise from the different cell types used.

Several investigations involving various cell types such as myocytes [13,14] or even Sertoli cells [31,46] suggest the involvement of GPCR in the generation of testosterone-induced signaling. In GC-2 cells DHEAS activation of the Src/Ras/Raf/Erk1/2 signaling module, leading to CREB and ATF-1 activation, is mediated by GPCR interacting with Gnα11 [28]. The similarities between DHEAS- and testosterone-induced signaling prompted us to investigate a possible involvement of Gnα11 in the actions of testosterone. The results obtained clearly demonstrate the participation of this protein in the generation of the non-classical testosterone pathway. Silencing of the expression of Gnα11 leads to the complete abolition of testosterone-induced stimulation of Erk1/2, ATF-1, or CREB demonstrated in immunofluorescence experiments (Fig. 7) and in western blots (Figs. 8 and 9). We therefore have to assume the existence of a membrane-bound GPCR for testosterone as the mediator of the non-classical testosterone signaling. Our conclusion is in a good agreement with various other studies proposing GPCR as mediators of the so-called non-genomic effects of steroid hormones. A series of recent investigations unveiled a membrane-bound GPCR for estrogen from the group of orphan receptors, referred to as GPER-1 [7,47]. Until these data were published, the classical cytosolic/nuclear estrogen receptors ERα and ERβ were thought to mediate both genomic and non-genomic effects of estrogen. Similarly, the new olfactory receptor family member PSGP (prostate-specific G-protein-coupled receptor) has been identified as a receptor for the testosterone metabolite 6-dehydrotestosterone [48]. The identification of steroid hormone-specific GPCRs such as GPER-1 or PSGP, which is predominantly expressed in prostate cancer cells, however, opens new avenues for investigation of the role of estrogens or androgens in organism physiology. By analogy, we think that the study presented here, which clearly shows the involvement of Gnα11 in the testosterone-induced non-classical signaling pathway, and further work focussing on the identification of the membrane-bound GPCR for testosterone will help to complete our knowledge concerning the action of steroid hormones. It may also help to distinguish between long-term genomic effects associated with the classical testosterone pathway that lead to sexual maturation and effects of the non-classical testosterone pathway that lead to rapid and perhaps transient responses to extracellular stimuli.

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