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Dehydroepiandrosterone sulfate mediates activation of transcription factors CREB and ATF-1 via a Gα11-coupled receptor in the spermatogenic cell line GC-2

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ABSTRACT

Dehydroepiandrosterone sulfate (DHEAS) is a circulating steroid produced in the adrenal cortex, brain, and gonads. Whereas a series of investigations attest to neuroprotective effects of the steroid in the brain, surprisingly little is known about the physiological effects of DHEAS on cells of the reproductive system. Here we demonstrate that DHEAS acting on the spermatogenic cell line GC-2 induces a time- and concentration-dependent phosphorylation of c-Src and Erk1/2 and activates the transcription factors activating transforming factor-1 (ATF-1) and cyclic AMP-responsive element binding protein (CREB). These actions are consistent with the non-classical signaling pathway of testosterone and suggest that DHEAS is a pro-androgen that is converted into testosterone in order to exert its biological activity. The fact, however, that steroid sulfatase mRNA was not detected in the GC-2 cells and the clear demonstration of DHEAS-induced activation of Erk1/2, ATF-1 and CREB after silencing the androgen receptor by small interfering RNA (siRNA) clearly contradict this assumption and make it appear unlikely that DHEAS has to be converted in the cytosol into a different steroid in order to activate the kinases and transcription factors mentioned. Instead, it is likely that the DHEAS-induced signaling is mediated through the interaction of the steroid with a membrane-bound G-protein-coupled receptor, since silencing of Guanine nucleotide-binding protein subunit alpha-11 (Gnα11) leads to the abolition of the DHEAS-induced stimulation of Erk1/2, ATF-1, and CREB. The investigation presented here shows a hormone-like activity of DHEAS on a spermatogenic cell line. Since DHEAS is produced in male and female reproductive organs, these findings could help to define new roles for DHEAS in the physiology of reproduction.

1. Introduction

Dehydroepiandrosterone (DHEA) is mainly produced by the adrenal zona reticularis and is almost entirely converted by the enzyme sulfotransferase to dehydroepiandrosterone sulfate (DHEAS), which is then secreted into the serum [1]. DHEAS is the most abundant circulating steroid. Its concentration in plasma is between 1.3 and 6.8 μM, which is approximately 200-fold higher than the plasma concentrations of DHEA (7–31 nM) [2].

While sulfated steroids have long been considered to be biologically inactive waste products of steroid hormone metabolism, the discovery of cytosolic steroid sulfatase prompted the new idea that the sulfates constitute a reservoir that upon desulfation can deliver precursors for steroid hormone synthesis. Thus, DHEAS has been viewed as a pro-androgen that, after being transported into cells, becomes desulfated by steroid sulfatase to DHEA and further converted into testosterone or other steroid hormones in order to exert its biological activity [3]. DHEA and DHEAS are also produced in the brain [4], where their biological activity is considered to be neuroprotection [5].

Numerous recent investigations demonstrate DHEAS-specific effects that are distinct from effects induced by DHEA, indicating that desulfation and conversion of DHEAS to other steroid hormones are not prerequisites for its actions and suggesting that caution should be used in interpreting the actions of either of the steroids. Thus, 1 μM DHEAS was shown to inhibit nerve growth factor (NGF)-induced proliferation of pheochromocytoma PC12 cells and to stimulate chromogranin A expression and catecholamine release from NGF-treated cells [6,7]. Similarly, DHEAS was shown to specifically stimulate growth factor-induced proliferation of bovine chromaffin cells in an age-dependent manner [8]. In the same investigation DHEA decreased the proliferative effect of the growth factors, indicating that the cellular responses to DHEA and DHEAS are mediated via different receptors [8]. Concerning their neuroprotective effects [5], DHEA and DHEAS might act by triggering different pathways. Thus, DHEA, but not DHEAS, prevented neurotoxicity induced by N-methyl-D-aspartate (NMDA) by inhibiting the NMDA-induced activation of Ca^{2+}-sensitive nitric oxide (NO) synthase and NO production [9]. In contrast, the neuroprotective effects of DHEAS against NMDA-

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induced cytotoxicity are most likely mediated through the Sig-1R receptor [9].

All of the above information indicates a role for DHEAS that is different from that of DHEA. Taking into consideration that DHEA and DHEAS are produced not only in the adrenal cortex and brain but also in the gonads [10–12], it is rather surprising that very little is known about the effects of DHEAS on the cells of the male or female reproductive systems. Thus, in order to investigate a possible biological significance of DHEAS in cells of the reproductive system we analyzed its effects on the spermatogenic cell line GC-2 spd (ts). The results obtained here reveal new aspects of DHEAS action and will possibly provide new insights into DHEAS-mediated physiological mechanisms associated with fertility and reproduction.

2. Materials and methods

2.1. Cell culture

The spermatogenic cell line GC-2 spd (ts) [13] (hereafter referred to as GC-2) was cultured as recommended in DMEM (TS) high glucose (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin combination (100 U/ml of each) and 1% L-glutamine. 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 (the 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. Various concentrations of DHEAS dissolved in ethanol were added to the cells and incubation was continued for various times (see figure legends for details). The concentration of ethanol was identical in all samples. The medium was then removed by aspiration and cells were washed twice with ice-cold phosphate-buffered saline (PBS; without Ca^{2+} or Mg^{2+}; PAA Laboratories GmbH) 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 20 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, Rockford, IL, 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 10 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 molecular weight markers (Cell Signaling Technology) were used to determine the relative molecular mass of the separated proteins. After electrophoresis proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for 30 min at 200 mA. Desired protein bands were visualized by incubating the membranes according to the protocol of the providers of the primary antibody (Table 1) and the appropriate secondary antibody of the enhanced chemiluminescence kit (ECL; GE HealthCare, Munich, Germany). For the simultaneous detection of phospho-CREB and phospho-ATF-1, western blots were probed with an antibody that cross-reacts 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.

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-phosho-Erk1/2 (for WB and IF)	4370	Cell Signaling Technology	Frankfurt am Main, Germany
Anti-phospho-c-Src (for WB)	4276	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
Anti-phospho-ATF-1 (for IF)	2456-1	Epitomics	Burlingame, USA

The chemiluminescence obtained was visualized by exposure to film. Films were analyzed by the TotalLab gel image analysis software (Biostep, Jahnsdorf, Germany).

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

Total mRNA was isolated from GC-2 cells by following the protocol of the commercially available RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and PCR amplification of steroid sulfatase-specific mRNA/cDNA were carried out by the Reverse Transcription System (Promega, Mannheim, Germany) according to the protocol of the provider. 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, 1–2.5 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 temperatures ranging between 60 and 62 °C for 1 min, and cDNA extension at 72 °C for 1 min. After amplification, a final extension at 72 °C was performed for 10 min. The forward primer was the oligonucleotide 5′ACTGCTTCCTCATG GACGACCTC3′ and the reverse primer was 5′AGGCGTTGCAGTAGTG GAACAGG3′. These amplify a region between bases 1001 and 1624 of mouse steroid sulfatase-specific mRNA and yield an amplificate of 624 bp.

GAPDH-specific mRNA/cDNA was detected using a similar protocol with the exceptions that the annealing temperature was kept constant at 54 °C, the extension time was 45 s, and the $MgCl_2$ concentration was 2.5 mM. The forward primer was the oligonucleotide 5′GGAGATTGTTGC CATCAACG3′ and the reverse primer 5′CACAATGCCAAAGTTGTCA3′. These 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′GTG GGGCTGCCAGCATTGGA3′, 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

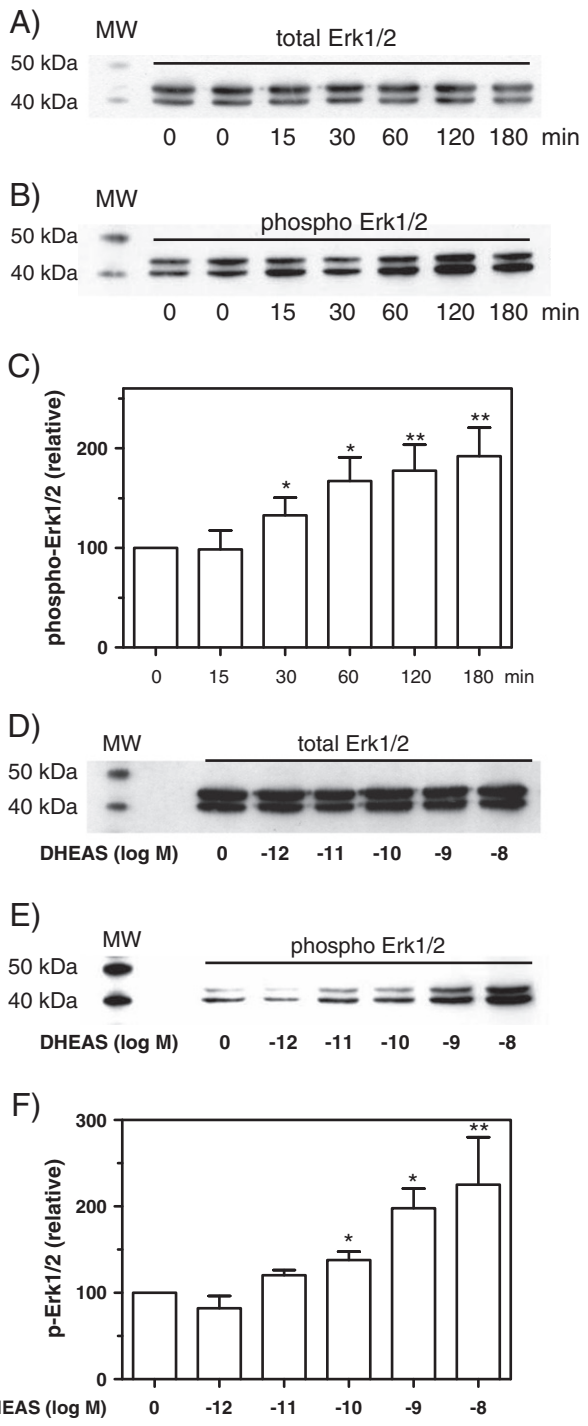

Fig. 1. Activation of Erk1/2 by DHEAS. Lysates of DHEAS-treated GC-2 cells were run on SDS-PAGE and subsequently probed in western blots. (A–C) Time-dependent activation of Erk1/2: While incubation with 1 nM DHEAS had no effect on the amount of total Erk1/2 (A), it stimulated its phosphorylation (B). Phosphorylation of Erk1/2 (corrected for the amount of total Erk1/2 as shown in panel A) was significant after 30 min of incubation with DHEAS (C) ($n = 4$; means ± SEM; * = $p \leq 0.05$; ** = $p \leq 0.01$). (D–E): Treatment of cells for 30 min with the indicated concentrations of DHEAS had no effect on total Erk1/2 (D) but led to an increase in phosphorylated Erk1/2 (E). Within this time frame activation of Erk1/2 (corrected for the amount of total Erk1/2 as shown in panel D) was significant at DHEAS concentrations ≥0.1 nM (C) ($n = 5$–7; means ± SEM; * = $p \leq 0.05$; ** = $p \leq 0.01$).

5′GAACCGGGAAGAGGTAGGG3′ and 5′GACCAAGTGTGAGTGCAGGA3′, respectively. These amplify a 917-bp fragment of mouse Gq11-specific mRNA localized between bases 70 and 986.

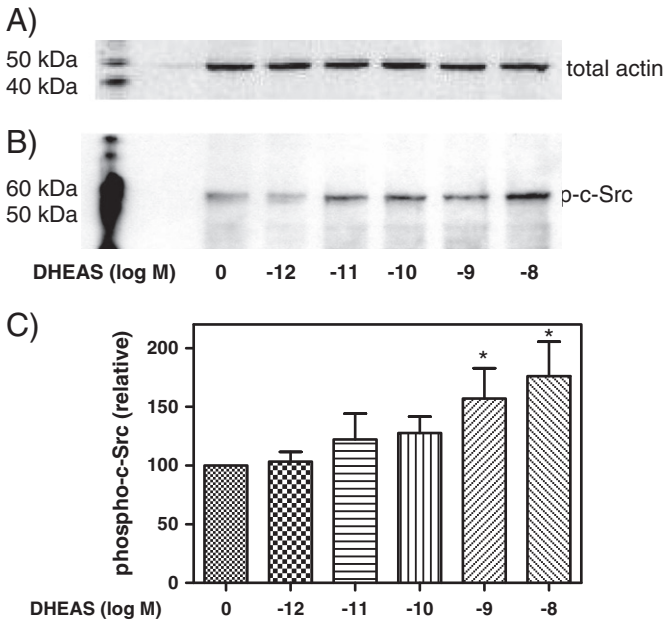

Fig. 2. Activation of c-Src by various concentrations of DHEAS. Conditions were the same as in Fig. 1D–E. Detection of total actin served as loading control (A). DHEAS stimulated c-Src phosphorylation (activation) at Tyr419 (B). Activation was significant at DHEAS concentrations ≥1 nM (B) ($n = 4$–5; means ± SEM; * = $p \leq 0.05$). Values in panel C were corrected for differences in loading by standardizing to the amount of total actin detected in parallel western blots (A).

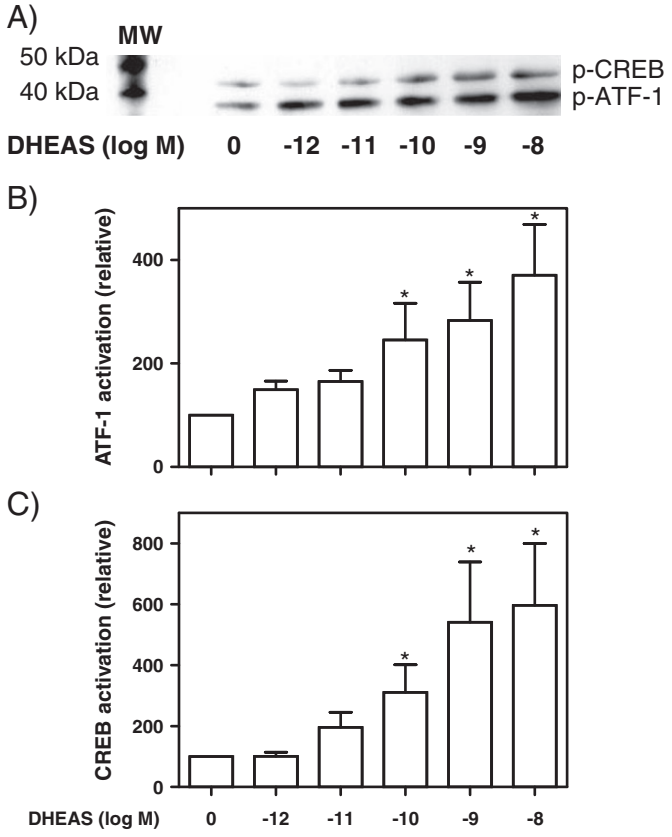

Fig. 3. DHEAS-induced activation of ATF-1 and CREB. Conditions were the same as for the experiments shown in Fig. 1D–E. For the western blot (A) an antibody against phospho-CREB was used that cross-reacts with phospho-ATF-1 (see "Materials and methods"). Activation of ATF-1 (B) or CREB (C) was significant at DHEAS concentrations ≥0.1 nM ($n = 4$; means ± SEM; * = $p \leq 0.05$). Values were corrected for differences in loading by standardizing to the amount of total actin detected in parallel western blots (as in Fig. 2).

2.5. Inhibition of steroid sulfatase by STX64

Cells were incubated as described above with or without 10 nM DHEAS in the presence or absence of 10 nM STX64 (Sigma-Aldrich, Taufkirchen, Germany). This concentration of STX64 has been considered sufficient for complete inactivation of steroid sulfatases [14]. All samples contained 2 μl DMSO, which was the solvent for stock preparations of STX64. After 30 min of incubation, cell lysates were prepared as described above. Activated Erk1/2 and total Erk1/2 were detected after SDS-PAGE and western blotting as stated under "Materials and methods", Section 2.3.

2.6. Silencing of the androgen receptor via siRNA

Silencing of the androgen receptor was carried out by using commercially available siRNA and by following the protocol of the provider (Stealth™ RNAi; Invitrogen, Karlsruhe, Germany). The oligonucleotides used were: primer pair 1: 5′ACUCGAUCGCAUCAUUGCAUGCAAA3′ and 5′UUUGCAUGCAAUGAUGCGAUCGAGU3′; primer pair 2: 5′CCCAGAAGAUGACUGUALJCACACAU3′ and 5′AUGUGUGAUACAGUCAUCUUCUGGG3′; and primer pair 3: 5′CCAGAUUCCUUUGCUGCCUUGUUAU3′ and AUAACAAGGCAGCAAAGGAAUCUGG3′. Control cells were treated with Opti-MEM plus Lipofectamine 2000 plus Stealth™ RNAi negative control. Transfection efficiency was estimated by the Block-iT™ Transfection Kit (Invitrogen, Karlsruhe, Germany) according the protocol of the provider. After 72 h of incubation cells were used to isolate mRNA for RT-PCR (see previous paragraph). A second set of cells was stimulated with 1 nM DHEAS 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 DHEAS and used for the isolation of cell lysates to be investigated in western blots.

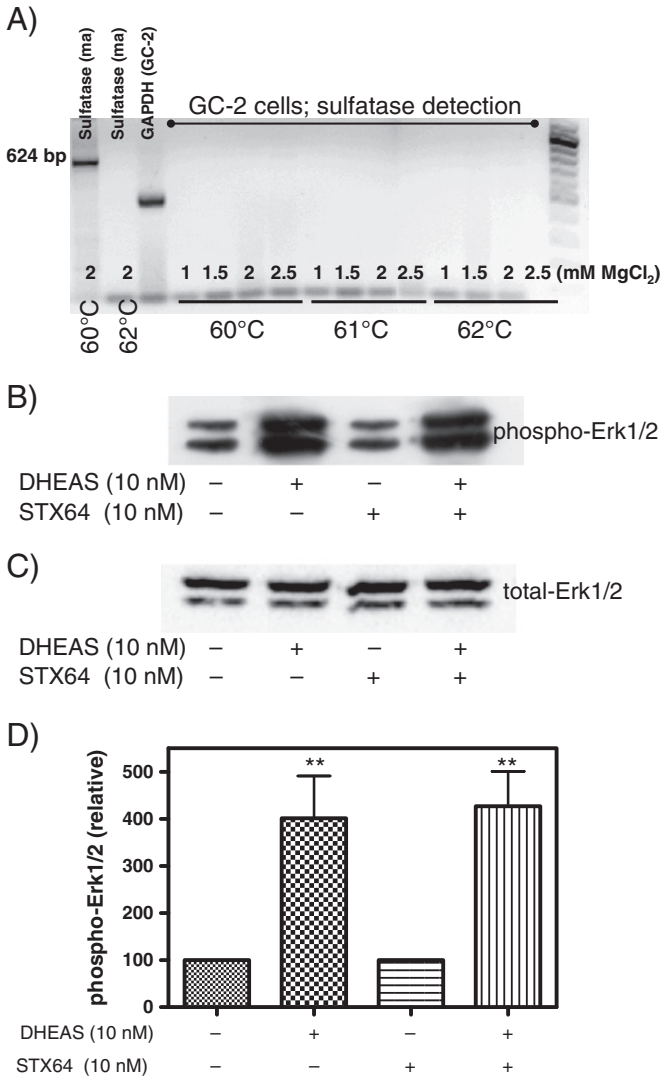

Fig. 4. Assessment of the involvement of steroid sulfatase in the DHEAS-induced signaling. (A) RT-PCR for the detection of steroid sulfatase-specific mRNA/cDNA. Steroid sulfatase-specific mRNA/cDNA was not detectable in GC-2 cell extracts at any annealing temperature and $MgCl_2$ concentration tested. The expected sulfatase-specific amplificate of 624 bp was clearly detected, however, in extracts from mouse adrenals, indicating the correct choice of primers. A GAPDH-specific amplificate was detected in the GC-2 preparations, indicating that the reason for not detecting sulfatase-specific amplificates in the GC-2 preparations was not due to the poor quality of the mRNA/cDNA employed. (B) Western blot demonstrating that the steroid sulfatase-specific inhibitor STX64 does not prevent or reduce DHEAS-induced stimulation of Erk1/2. (C) Western blot showing total Erk1/2 in the same cell lysates used in panel B. (D) DHEAS-induced activation of Erk1/2 in the presence STX64 was highly significant ($n = 3$; means ± SEM; ** = $p \leq 0.01$).

2.7. Silencing of the Gnα11 protein via siRNA

Silencing of the Gnα11 protein was carried out by using commercially available siRNA and by following the protocol of the provider (Silencer® Select siRNA; Invitrogen). The oligonucleotides used were: primer pair 1: 5′CCAAGUUGGUGUACCAGAAtt3′ and 5′UUCUGGUACACCAACUUGGtg′; and primer pair 2: 5′CAAGAUCCUCUACAAGUAUtt3′ and 5′AUACUUGUAGAGGAUCUUGag3′. Control cells were treated with Opti-MEM plus Lipofectamine 2000 plus the siRNA negative control as supplied by the provider. All other steps were the same as described in the previous paragraph.

2.8. Immunofluorescence

GC-2 cells that had been treated with siRNA to silence either AR or Gα11 were incubated with vehicle alone or vehicle + 1 nM DHEAS 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 2 days at 4 °C in a humidified chamber. The antibody against phospho-Erk1/2 was from Cell Signaling Technology. The antibody against phospho-ATF-1 was from Epitomics (Burlingame, CA, USA). This antibody is phospho-ATF-1 specific and does not interact with phospho-CREB. For the specific detection of phospho-CREB, an antibody from Cell Signaling Technology was used with

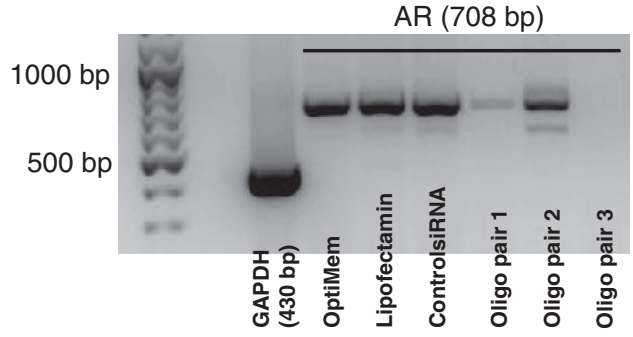

Fig. 5. Silencing expression of AR by means of siRNA. GC-2 cells were treated with three different nucleotide pairs of siRNA against AR according to the manufacturer's protocol. Control cells were treated with either Opti-MEM alone, Opti-MEM plus Lipofectamine, or both of the above plus negative control siRNA, provided in the kit of the manufacturer. 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 siRNA oligo pair 3 abolished the expression of AR-specific mRNA/cDNA. This oligo pair was used for the subsequent experiments depicted in Fig. 6 and 7.

negligible interaction with phospho-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/). All cells in the optical field were considered. Data points were transferred to and analyzed by the software program GraphPad Prism4 (GraphPad Software, Inc., La Jolla, CA, USA).

2.9. 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 bands, detected in western blots that were run

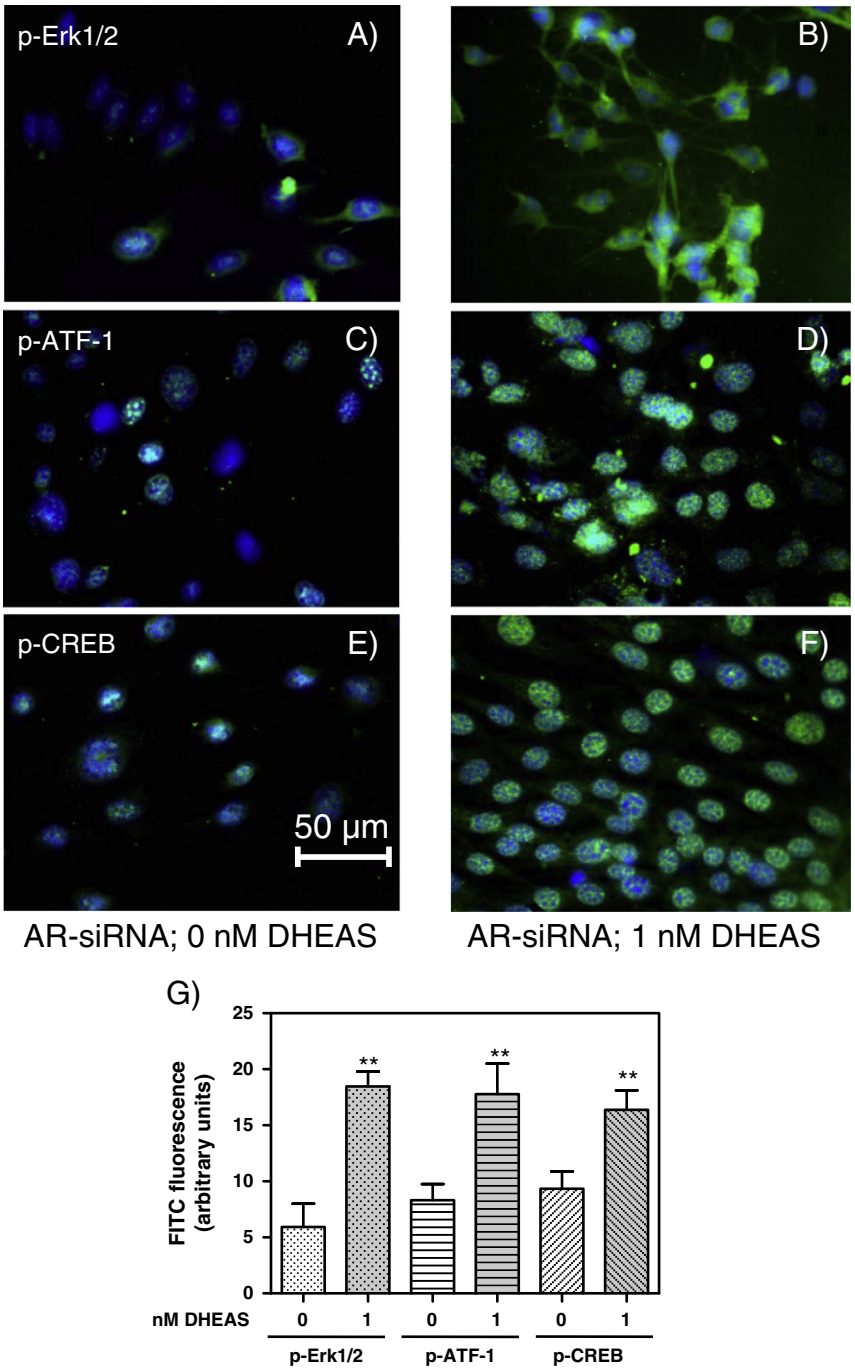

Fig. 6. Detection of phospho-Erk1/2, phospho-ATF-1 and phospho-CREB by immunofluorescence after silencing AR expression by siRNA. All cells shown were treated with oligo pair 3 to silence AR expression. Nuclei were stained with DAPI and with specific primary antibodies against phospho-Erk1/2, phospho-ATF-1 or phospho-CREB and an Alexa Fluor 488-labeled secondary antibody as detailed in "Materials and methods". Treatment of the GC-2 cells with 1 nM DHEAS for 30 min triggered the activation of Erk1/2 (B), ATF-1 (D) and CREB (F). The fluorescence signals indicating activated Erk1/2, CREB, or ATF-1 were significantly higher (G) after 30 min of incubation with 1 nM DHEAS than the signals measured in the absence of the steroid (A, C, E) (n = 19–28; means ± SEM; ** = $p \leq 0.01$; scale bar = 50 μm).

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. DHEAS induces activation of Erk1/2

Steroid hormones exert their non-classical actions by activating enzymes of signaling cascades that are usually triggered by growth factors [15,16]. One of these is the Src/Ras/Raf/Erk1/2 signaling cascade, and thus our first aim was to examine whether DHEAS might induce Erk1/2 activation in GC-2 cells.

As shown in Fig. 1B, 1 nM DHEAS induced a clear activation (phosphorylation) of Erk1/2 that was significant after 30 min of incubation (Fig. 1C). When Erk1/2 activation was determined by incubating the cells for 30 min with various concentrations of DHEAS (Fig. 1E), significant activation of Erk1/2 was obtained at DHEAS concentrations of 0.1 nM and above (Fig. 1F). Incubation with DHEAS did not affect expression of total Erk1/2 (Fig. 1A and D).

In parallel experiments we addressed the effect of DHEA on Erk1/2 activation under conditions identical to those used for the investigation of the DHEAS effect. DHEA-induced activation was never observed in these experiments; in fact, if anything there was a small but significant reduction in active Erk1/2 seen with 10 nM DHEA (see Supplementary material). After obtaining this result, we confined our further investigations to DHEAS-induced signaling.

3.2. c-Src activation by DHEAS

Steroid hormone-induced Erk1/2 activation has been shown in several cases to be mediated by activation of c-Src [17–20] via phosphorylation at Tyr419. A similar mechanism is apparently involved in the DHEAS-stimulated induction of signaling cascades in GC-2 cells: we found that DHEAS stimulates phosphorylation of c-Src at Tyr419, which was visualized by western blotting using a monoclonal antibody specifically recognizing the phosphorylated form of this amino acid (Fig. 2A). After 30 min of incubation with various concentrations of DHEAS, significant activation of c-Src was obtained at concentrations of 1 nM or greater (Fig. 2B).

3.3. DHEAS-induced activation of transcription factors CREB and ATF-1

Activation of Erk1/2 leads to its translocation to the nucleus and to subsequent activation of transcription factors. Stimulation of the c-Src/Ras/c-Raf/Erk1/2 pathway is known to activate the transcription factors CREB (cyclic AMP-responsive element binding protein) and ATF-1 (activating transforming factor-1) [16,18,21]. Since the results shown in Fig. 1 and 2 clearly demonstrate activation of this signaling cascade, we investigated a possible DHEAS-induced activation of ATF-1 and CREB in the GC-2 cells. In western blots with an antibody that cross-reacts with phospho-CREB and phospho-ATF-1, we observed significant activation of both transcription factors following 30 min of incubation with 0.1 nM DHEAS (Fig. 3A–C). This response was similar to that observed for the DHEAS-induced activation of Erk1/2 (Fig. 1F).

3.4. Lack of steroid sulfatase expression in GC-2 cells

DHEAS is often considered a pro-androgen that needs to be desulfated to DHEA and thereafter converted to testosterone in order to exert its androgenic properties [3]. Therefore, we examined whether the GC-2 cells might express steroid sulfatase-specific mRNA. Sulfatase-specific mRNA/cDNA was not detectable at all annealing temperatures and $MgCl_2$ concentrations used (Fig. 4A). In the same preparations GAPDH was detectable (Fig. 4A), indicating that mRNA isolation and its reverse transcription to cDNA had been carried out correctly and a faulty RT-PCR could not be the reason for the lack of sulfatase-specific amplificates in extracts of GC-2 cells. The expected sulfatase-specific amplificate of 624 bp was clearly present, however, in extracts from mouse adrenals at an annealing temperature of 60 °C in the presence of 2 mM $MgCl_2$, indicating that the lack of signals in extracts of GC-2 cells was not due to flawed primers (Fig. 4A).

To further confirm that steroid sulfatase is not involved in the generation of DHEAS-induced signaling, we used the steroid sulfatase-specific inhibitor STX64 and investigated the effects of DHEAS on activation of Erk1/2 in the presence of this compound. As Fig. 4B shows, 10 nm STX64 did not inhibit DHEAS-induced phosphorylation of Erk1/2. Total Erk1/2 was not influenced under these conditions (Fig. 4C). The stimulation of Erk1/2 phosphorylation under these conditions was highly significant (Fig. 4D).

The implication of the experiments summarized in Fig. 4 is that DHEAS does not exert its effects via conversion to DHEA but rather directly, by binding to a receptor in a hormone-like fashion.

3.5. Does the androgen receptor mediate the DHEAS effects?

The results described in the previous paragraphs clearly show a strong overlap between DHEAS-induced signaling and the non-classical action of testosterone [16]. Thus, one can speculate on a possible involvement of the androgen receptor in DHEAS-induced signaling. This possibility was addressed by re-investigating DHEAS effects on Erk1/2, CREB and ATF-1 activation after silencing the AR expression by means of siRNA.

Fig. 5 shows the RT-PCR results obtained after attempting to silence the expression of AR-specific mRNA by using 3 different oligonucleotide pairs. It is apparent that the best result was obtained by using the third combination of oligonucleotide primers (oligo pair 3), whose sequence was listed under "Materials and methods". The expression of AR-specific mRNA was not affected by either Lipofectamine or control siRNA (Fig. 5). Thus, for the following experiments cells were treated with oligo pair 3.

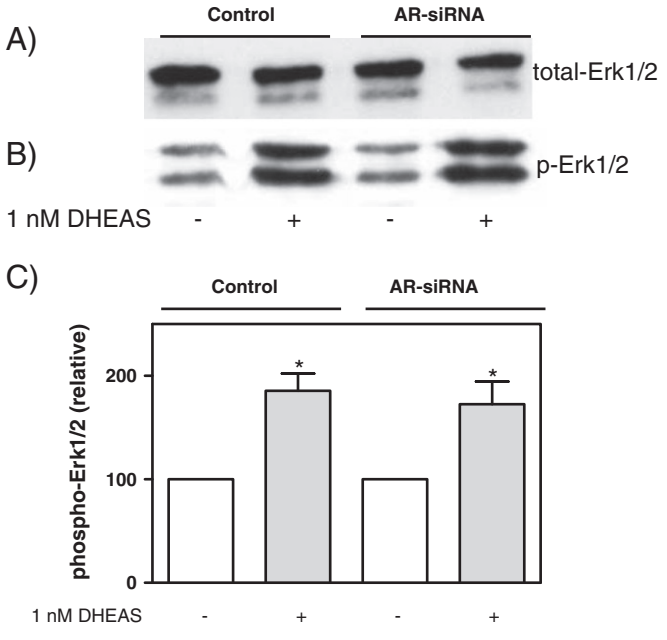

Fig. 7. Western blot analysis of phospho-Erk1/2 after silencing AR expression by siRNA. Cells were treated with control siRNA or with siRNA to silence AR expression. After 30 min of incubation in the presence or absence of 1 nM DHEAS, cell lysates were prepared and probed in a western blot as described under "Materials and methods". Incubation with 1 nM DHEAS, which had no effect on total Erk1/2 (A), triggered the formation of phospho-Erk1/2 independent of whether the AR had been silenced or not (B, C). The data shown in (C) were corrected for the amount of total Erk1/2 as shown in (A) ($n = 3$; means ± SEM; * = $p \leq 0.05$).

Cells were incubated with either 0 or 1 nM DHEAS for 30 min and then subjected to a fixation/immunostaining procedure as described under "Materials and methods" to detect the phosphorylated forms of either Erk1/2, ATF-1, or CREB. Fig. 6 shows that treatment of the GC-2 cells with AR-specific siRNA does not affect the DHEAS-induced activation (phosphorylation) of Erk1/2, ATF-1 or CREB. Erk1/2 activation is seen in the form of green fluorescence that is spread over the entire volume of DHEAS-treated cells despite the absence of AR (Fig. 6B). Activated transcription factors ATF-1 (Fig. 6D) and CREB (Fig. 6F) are visible as green fluorescent signals within the nucleus after the exposure of the cells to DHEAS. In the absence of DHEAS, a relatively low, basal amount of activated Erk1/2 (Fig. 6A), ATF-1 (Fig. 6C), or CREB (Fig. 6E) was restricted to a few cells only. Total cell-associated fluorescence corresponding to active Erk1/2, ATF-1, or CREB was significantly higher after 30 min of incubation with 1 nM DHEAS than the fluorescence measured in the absence of the steroid (Fig. 6G; see also upper "control" panels of Fig. 10A–C for the respective control responses to DHEAS in the absence of any siRNA, which were similar to the responses shown in Fig. 6). Since immunofluorescence considers only cells residing within the optical field of the microscope, we carried out an additional western blot experiment in order to obtain an average for all cells in the incubation mixture. As can be seen in Fig. 7, treatment of GC-2 cells with siRNA to silence the expression of AR does not impair the ability of DHEAS to induce Erk1/2 activation, which is consistent with the results shown in Fig. 6A and B.

The information shown in Figs. 5, 6, and 7 indicates that the AR is not involved in the generation of DHEAS-induced signaling. Nevertheless, since silencing of mRNA might not necessarily lead to a rapid decrease in the expression of the targeted protein, we investigated by immunofluorescence whether the AR protein is still present in the cells despite the reduction of AR-specific mRNA/cDNA by siRNA. The results of the investigation are summarized in Fig. 8. While green fluorescence, indicating the expression of the AR protein, is visible in every GC-2 cell in the image shown in Fig. 8A, it is entirely missing when the first antibody against AR was omitted (Fig. 8B) or after cells were treated with siRNA (primer pair 3) to prevent expression of AR-specific mRNA (Fig. 8C).

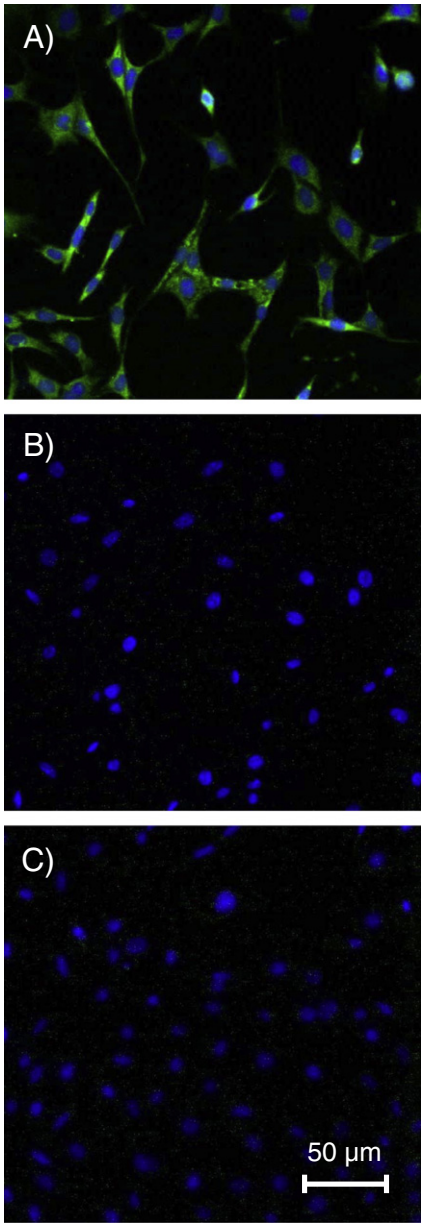

Fig. 8. Silencing AR protein expression by siRNA. Cells were fixed in methanol and incubated with a primary antibody against the AR and a fluorescent secondary antibody (rabbit anti-goat IgG-FITC green), as described under "Materials and methods". Nuclei are stained by DAPI, as described under "Materials and methods". (A) All control cells show green fluorescence, indicating the presence of the AR. (B) When the primary antibody was omitted, only DAPI-stained nuclei were visible, indicating that the green fluorescence seen in (A) was not due to non-specific binding of the secondary antibody. (C) When cells were treated with siRNA to silence AR expression, AR-specific protein was not detectable by the combination of the antibodies used in (A), indicating the successful silencing of the AR protein. Only DAPI-stained nuclei can be seen in (B) and (C).

3.6. Involvement of Gnα11 in DHEAS-induced signaling

Many hormones, among them steroid hormones, elicit their actions through G-protein-coupled receptors (GPCRs) [22–25]. In the mast cell line RBH-2H3 the Gq/11 protein was shown to interact with DHEAS [26]. Although there appear to be no reports concerning the expression of Gq/11 in the various cell types of mouse testes, Gnα11 (equivalent to Gq/11) expression was detected in all cell types of human testes [27]. Since we determined that the AR does not participate in DHEAS-induced signaling in GC-2 cells, and because GC-2 cells express Gnα11 mRNA (Fig. 9), we investigated a possible involvement of GPCRs in the signaling cascade by silencing Gnα11 expression in these cells.

The RT-PCR results shown in Fig. 9 demonstrate that after transforming GC-2 cells with the siRNA oligo pair 2 (see Materials and methods), the expression of Gnα11-specific mRNA/cDNA is reduced to a minimum. The expression of Gnα11-specific mRNA was not affected by either Lipofectamine + Opti-MEM or control siRNA (Fig. 9). Oligo pair 1 caused only a slight reduction in the expression of Gnα11-specific

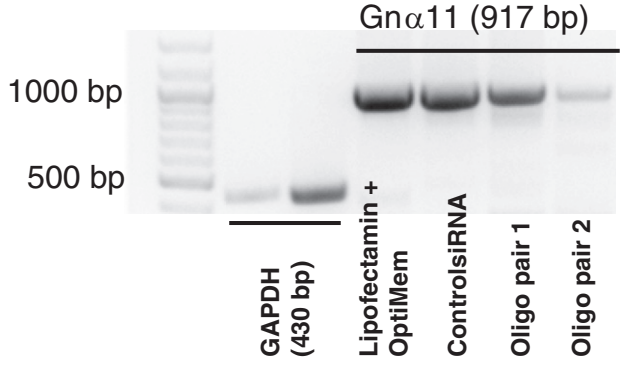

Fig. 9. Silencing expression of Gnα11-specific mRNA/cDNA by means of siRNA. Cells were incubated with 2 different oligonucleotide pairs (oligo pair 1 or 2) to silence Gnα11-specific mRNA expression. Isolation of mRNA and RT-PCR were carried out as described under "Materials and methods". Oligonucleotide pair 2 was the most efficient and was used in all subsequent experiments to silence the expression of Gnα11 (Fig. 10 and 11). Treatment of the cells with Opti-MEM plus Lipofectamine or Opti-MEM plus Lipofectamine plus the control siRNA (control siRNA) did not influence Gnα11 mRNA expression.

mRNA/cDNA. Thus, for the following experiments cells were treated with oligo pair 2.

GC-2 cells treated with either control siRNA or with siRNA against Gnα11 were incubated with 0 or 1 nM DHEAS for 30 min. Detection of phospho-Erk1/2, phospho-ATF-1, or phospho-CREB by immunofluorescence was carried out as described under "Materials and methods".

Treatment of GC-2 cells with control siRNA did not affect activation of Erk1/2, ATF-1, or CREB by 1 nM DHEAS (Fig. 10). Total cell-associated fluorescence corresponding to active Erk1/2, ATF-1, or CREB was significantly higher after 30 min of incubation with 1 nM DHEAS in cells treated with control siRNA than the fluorescence measured in the absence of the steroid. In parallel, treatment with siRNA against Gnα11 expression leads to the complete abolition of the effects of DHEAS, clearly demonstrating the involvement of this protein in mediating DHEAS-induced signaling. Fluorescence corresponding to active Erk1/2, ATF-1, or CREB after 30 min of incubation with 1 nM DHEAS was at the same level as the fluorescence measured in the absence of the steroid in these Gnα11-specific siRNA-treated cells.

The western blot shown in Fig. 11 confirms the immunofluorescence experiments. Silencing Gnα11 expression by transforming the GC-2 cells with oligo pair 2 leads to abolition of the DHEAS-induced activation of Erk1/2 (Fig. 11). Treatment of the cells with the negative control siRNA had no effect on Erk1/2 activation (Fig. 11), which at 1 nM DHEAS occurred to the same extent as in untreated cells (Fig. 1F).

4. Discussion

Steroid hormones are known to mediate their effects by two different mechanisms: In the so-called "classical" action of steroid hormones, they bind to intracellular steroid hormone receptors, which function essentially as ligand-activated transcription factors. Once activated, these receptors bind to DNA and activate the expression of target genes. In the

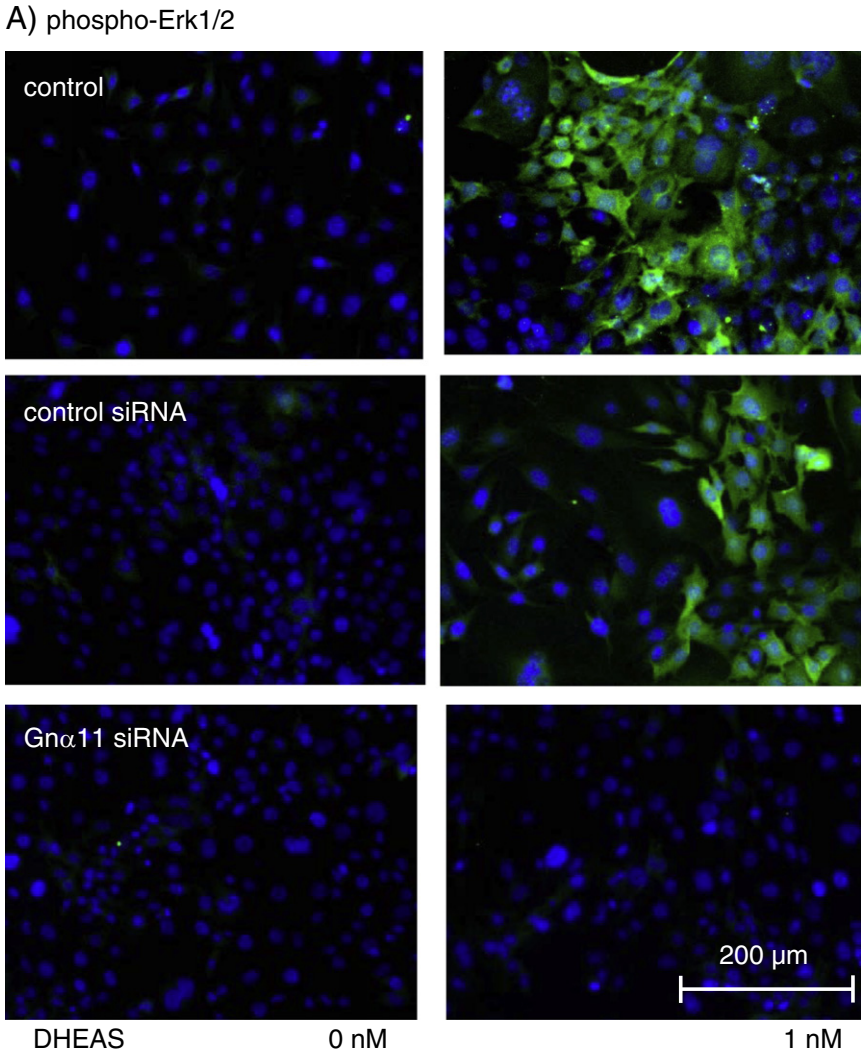

Fig. 10. Detection of phospho-Erk1/2, phospho-ATF-1 and phospho-CREB by immunofluorescence after silencing Gnα11 expression by siRNA. (A–C) Left columns: cells were treated with vehicle only (no DHEAS). Right columns: cells received 1 nM DHEAS. Upper rows: controls (untreated cells). Middle rows: controls treated with Lipofectamine, Opti-MEM, and negative control siRNA. Bottom rows: cells treated with Lipofectamine, Opti-MEM, and siRNA to silence Gnα11 expression (oligo pair 2). (A) Detection of activated Erk1/2. In the absence of DHEAS, few or none of the cells showed green fluorescence, indicating active Erk1/2. Control cells and cells treated with control siRNA responded to 1 nM DHEAS and displayed active Erk1/2 in almost all cells at various intensities. DHEAS did not induce Erk1/2 activation in cells that had been treated with siRNA to silence the expression of Gnα11. (B) Detection of activated ATF-1. When cells were treated with siRNA against Gnα11, DHEAS did not induce ATF-1 activation (green fluorescence within cell nuclei). In control cells and in cells treated with control siRNA, DHEAS induced ATF-1 activation in all nuclei. (C) Detection of activated CREB. DHEAS-induced activation of CREB (green fluorescence) was absent in nuclei of cells treated with siRNA to silence Gnα11 expression. Control cells and cells treated with negative control siRNA displayed active CREB in all nuclei after DHEAS treatment. (D) Statistical analysis of the results shown in panels A–C. The green fluorescence of all cells in the optical field was considered ($n = 70–170$; means ± SEM; ** = $p \leq 0.01$).

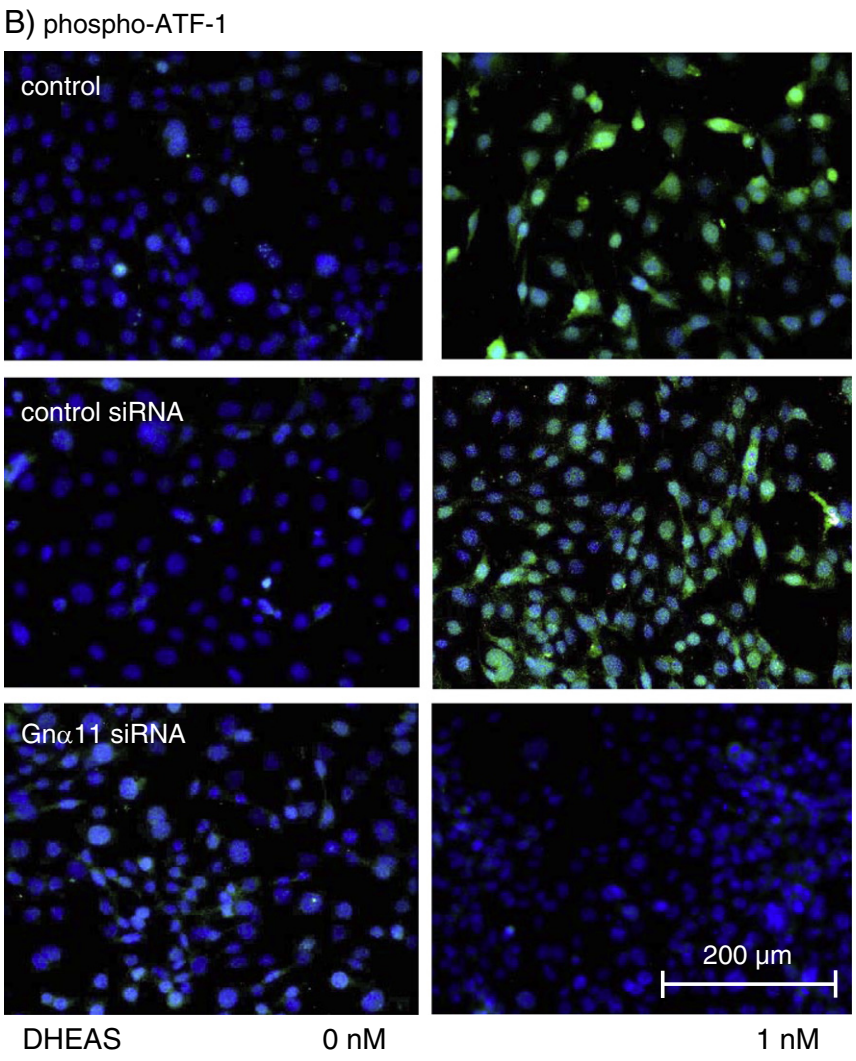

Fig. 10 (*continued*).

so-called "non-classical" pathway, steroid hormones bind to receptors associated with the plasma membrane [15]. These latter receptors are possibly localized within membrane rafts and mediate rapid activation of intracellular signaling cascades [28], which in some cases are identical to cascades normally activated by growth factors, such as the Src/PI3K/Akt or the Src/Ras/Raf/Erk1/2 pathways [15,29]. While DHEA has been shown to induce similar cascades in neuronal cells [30,31], little is known about the action of DHEAS, especially on cells of the reproductive system, although it has been shown to be produced in rodent gonads [32,33].

The process of spermatogenesis and the maturation of spermatogonia to spermatozoa depend on the activation of Erk1/2 and other mitogen-activated protein kinases (MAPK) [34,35], and Erk1/2 activation is an absolute requirement for the production of haploid spermatozoa [36,37]. Therefore, we first investigated whether DHEAS might induce Erk1/2 activation in a spermatogenic cell line. As shown in Fig. 1, DHEAS induces a significant activation (phosphorylation) of Erk1/2 in a time- and concentration-dependent manner in GC-2 cells. This is the first demonstration of Erk1/2 activation by DHEAS in a cell line derived from the reproductive system.

In accordance with the non-classical pathway of steroid hormone receptor action, Erk1/2 activation by DHEAS is accompanied by c-Src activation via phosphorylation at Tyr419 (Fig. 2). This result showing a link between c-Src and Erk1/2 activation is in good agreement with other studies demonstrating similar effects of steroid hormones [20,38] and suggests that DHEAS, consistent with it being a steroid hormone, triggers the c-Src/Ras/c-Raf/Erk1/2 signaling cascade. In Sertoli cells, the induction of this signaling pathway leads to the activation of the transcription factor CREB [16,18] and of the CREB-related factor ATF-1 [21]. Both CREB and ATF-1 are members of the bZIP superfamily of transcription factors and stimulate transcription when phosphorylated either at Ser133 (CREB) or at Ser63 (ATF-1), residues localized within a conserved region of the two proteins termed the phosphorylation box [39]. Transcription factors like CREB or ATF-1 that bind to cAMP-responsive element (CRE) promoters induce the transcription of a great variety of genes.

CREB and phospho-CREB are present not only in Sertoli cells but also in various other cells of the gonads, including spermatogonia, round spermatids, and, as shown recently, also in elongated spermatids [40–42]. CREB/CRE-inducible transcription is essential for the survival of spermatocytes and the production of mature spermatozoa [43]. The amount of phospho-CREB varies during the spermatogenic cycle [42], which would be consistent with it being directly involved in the differentiation process of germ cells.

The results summarized in Fig. 3 clearly show the DHEAS-induced activation of both CREB and ATF-1 in the spermatogenic cell line GC-2.

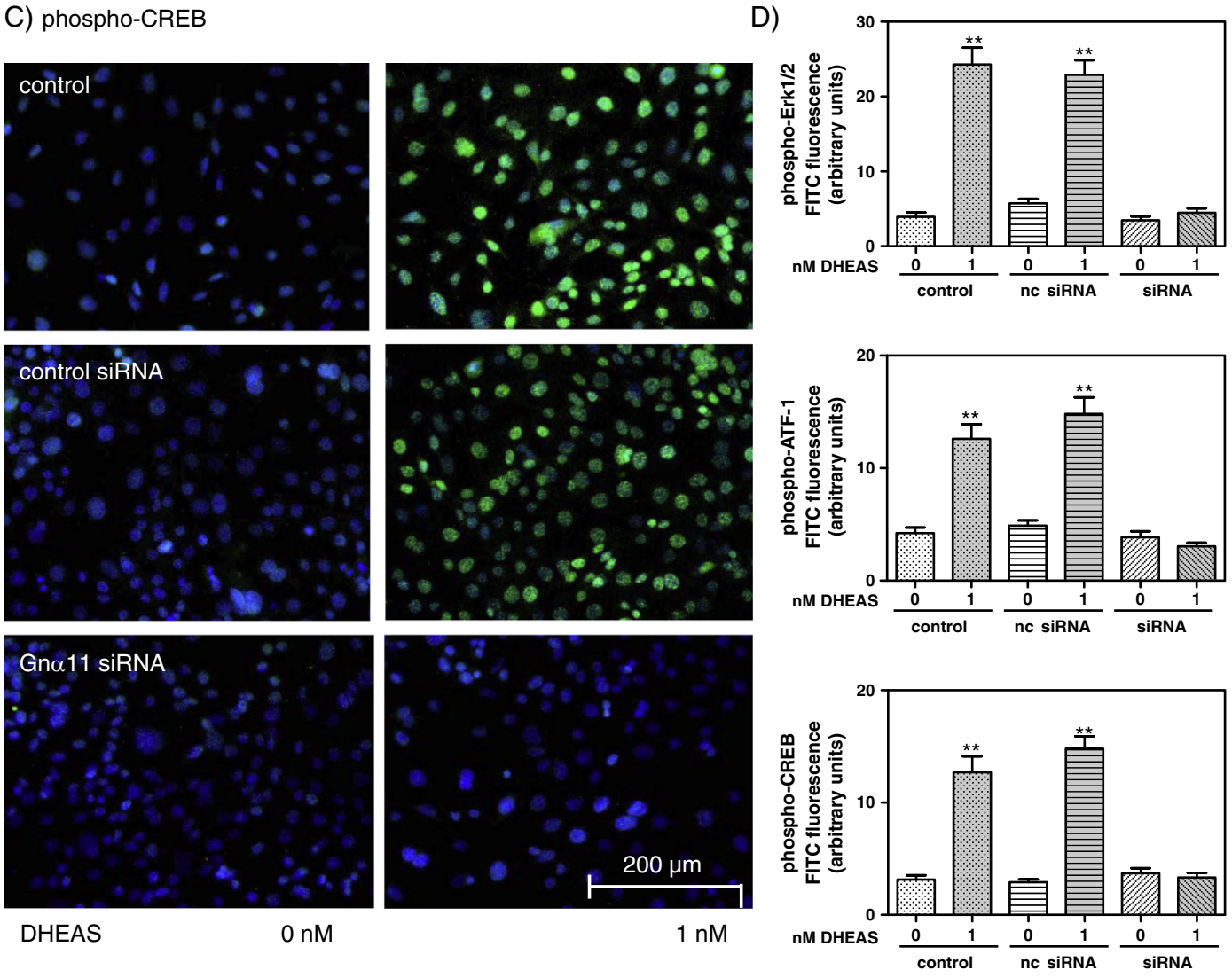

Fig. 10 (*continued*).

A significant activation of ATF-1 (Fig. 3B) or CREB (Fig. 3C) was obtained at DHEAS concentrations of 0.1 nM or greater, as was seen for the activation of Erk1/2 (Fig. 1F).

Although steroid sulfatase was not involved in the DHEAS-induced activation of Erk1/2 (Fig. 4), suggesting that DHEAS is not being converted to DHEA or testosterone to exert its effects, the fact that DHEAS induces a signaling cascade similar to the non-classical signaling pathway of testosterone [44] made it appear possible that the AR is involved in the propagation of the DHEAS-induced signaling. Therefore, we addressed a possible involvement of the AR in the generation of the DHEAS-induced signaling cascade in a series of experiments by restricting its expression at the mRNA and protein level by means of siRNA (Fig. 5 and 8). The results summarized in Fig. 6 and 7 clearly show that the abolition of AR does not affect the induction of the DHEAS-induced signaling cascade that leads to activation of Erk1/2, ATF-1, and CREB. This, together with the experiment showing the absence of steroid sulfatase, indicates that DHEAS must exert its effects by a different pathway which does not require desulfation or AR.

We next considered what other membrane-associated hormone receptors might mediate the observed effects of DHEAS. Owing to the fact that GPCRs have been shown to trigger activation of Erk1/2 in various signaling cascades [45–47] and because steroid hormones often mediate their actions through GPCRs [22–25], we investigated a possible involvement of a receptor coupled to a G-protein in DHEAS signaling by silencing the expression of Gnα11.

Gnα11 is a member of the Gqα family of heterotrimeric G proteins [47]. It is ubiquitously expressed across tissues and is present also in GC-2 cells, as shown in Fig. 9. Silencing Gnα11 expression in GC-2 cells abolished all DHEAS-induced signaling observed thus far: stimulation of Erk1/2, ATF-1, and CREB was no longer detected after treatment of the cells with Gnα11-specific siRNA, while the treatment of the cells with the control siRNA did not influence the DHEAS-induced activation of these enzymes and factors (Fig. 10 and 11).

The results presented here clearly indicate the involvement of a GPCR in the action of DHEAS and support earlier findings showing the involvement of Gq/11 in the actions of DHEAS on the mast cell-line RBL-2H3 [26]. Nevertheless, although GPCRs have been identified or proposed for various steroid hormones, the actual DHEAS-specific GPCR has yet to be identified.

In summary, our investigation calls into question the heretofore generally accepted idea of DHEAS being simply a pro-androgen and demonstrates for the first time that DHEAS acts as a steroid hormone on a spermatogenic cell line and triggers the activation of a signaling cascade that reflects the non-classical signaling pathway of steroid hormones

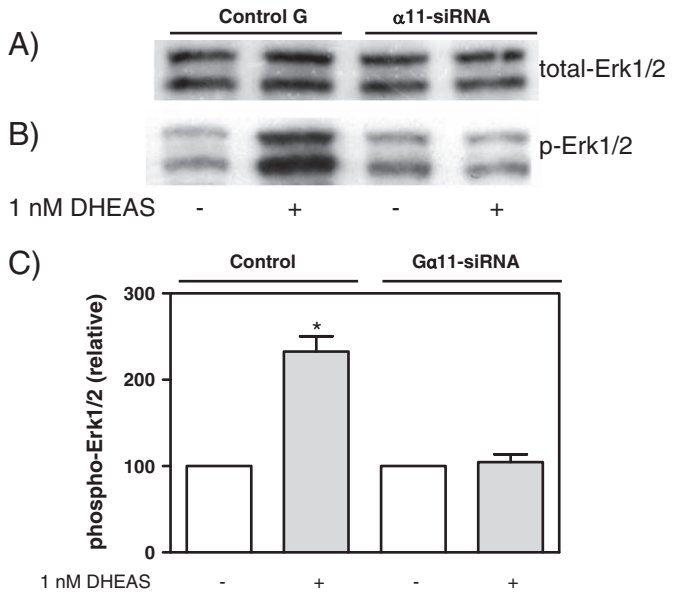

Fig. 11. Western blot analysis of phospho-Erk1/2 after silencing Gnα11 expression by siRNA. The preparation of cell lysates and the detection of proteins in western blots are described under "Materials and methods". (A) The amount of total Erk1/2 was not affected during the course of the incubation. (B) DHEAS stimulated Erk1/2 activation in GC-2 cells treated with negative control siRNA ("Control") but had no effect in cells that had been treated with siRNA to silence the expression of Gnα11. (C) For statistical analysis, data were corrected for the amount of total Erk1/2 as shown in panel (A) ($n = 3$; means ± SEM; * = $p \leq 0.05$).

involving membrane-bound GPCRs. The identification of the DHEAS receptor and of target mRNAs whose expression is controlled by the activation of the CRE promoters through the transcription factors CREB and ATF-1 will help to define a role of DHEAS in the physiology of cells of the male and possibly also of the female reproduction system.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.08.015.

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