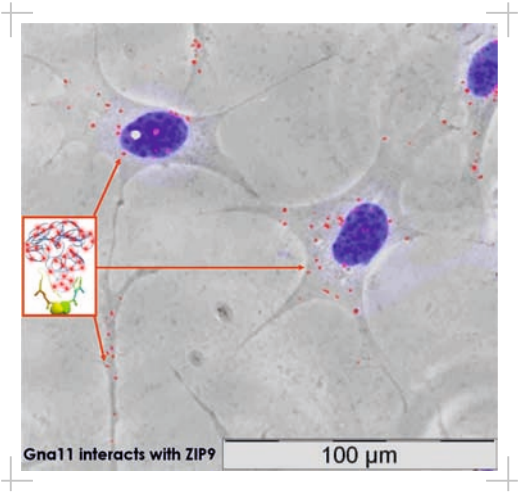
MAZEN SHIHAN

Identification of the G-protein Gnα11 and of the zinc transporter ZIP9 as key components in the mediation of non-classical signaling cascades of androgenic steroids



INAUGURAL DISSERTATION
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Faculty of Veterinary Medicine, Institute for Veterinary Physiology and Biochemistry
Justus-Liebig-University Giessen, Germany
Prof. Dr. rer. nat. Georgios Scheiner-Bobis

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submitted by

Mazen Shihan

Veterinarian from Hama, Syria

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Dean: Prof. Dr. Dr. h.c. Martin Kramer

Referee 1: Prof. Dr. rer. nat. Georgios Scheiner-Bobis

Referee 2: Prof. Dr. Gerhard Schuler

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Abbreviations

1. 3ß-HSD: 3beta-Hydroxysteroid dehydrogenase
2. AR: Androgen receptor
3. ATF-1: Activating transcription factor-1
4. CaMKII: Ca^{2+}/calmodulin kinase
5. cAMP: Cyclic adenosine monophosphate
6. c-Raf: Rapidly accelerated fibro sarcoma
7. CRE: cAMP response element
8. CREB: cAMP response element-binding protein
9. CREM: cAMP response element modulator
10. c-Src: Proto-oncogene sarcoma protein
11. DAG: Diacylglycerol
12. DHEA: Dehydroepiandrosterone
13. DHEAS: Dehydroepiandrosterone sulfate
14. DHT: Dihydrotestosterone
15. DMAPP: Dimethylallyl pyrophosphate
16. Erk1/2 (MAPK): Extracellular signal-regulated Kinase
17. ERß: Estrogen receptor beta
18. ERα: Estrogen receptor alpha
19. GC-2: Spermatogenic germ cell line
20. Gnα11 (Ga11): G protein, Alpha 11 (Gq Class)
21. GPCR: G protein-coupled receptor
22. GPER-1: Membrane-bound GPCR for Estrogen
23. GPP: Geranyl pyrophosphate
24. G-protein: Guanine nucleotide-binding proteins
25. Gα: Alpha subunit of G-protein
26. Gβ: Beta subunit of G-protein
27. Gγ: Gamma subunit of G-protein
28. Gα(i): Inhibitory subunit of G-protein
29. Gα(s): Stimulatory alpha subunit of G-protein

30. HMG-CoA: 3-Hydroxy-3-methylglutaryl-Coenzyme A
31. IP3: Inositol 1,4,5-trisphosphate
32. IPP: Isopentenyl pyrophosphate
33. MAPK: Mitogen-activated protein kinase
34. NGF: Nerve growth factor
35. NMDA: N-methyl-D-aspartate
36. NO: Nitric oxide
37. P450scc (CYP11A1): Side chain cleavage enzyme
38. PC12: Pheochromocytoma cell line
39. PIP2: Phosphatidylinositol 4,5-bisphosphate
40. PKA: Protein kinase A
41. PLC: Phospholipase C
42. PSGP: Prostate-specific G protein-coupled Receptor
43. RBL-2H3: Mast cell line
44. SHRs: Steroid hormone receptors
45. Sig-1R: Sigma-1/receptor
46. siRNA: Small interfering RNA
47. StAR (STARD1): Steroidogenic acute regulatory protein
48. STS: Steroid sulfatase
49. STX64: Irosustat ($C_{14}H_{15}NO_5S$)
50. SULT2A1: Cytosolic sulfotranferase
51. ZIP9: Zinc transporter

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I. 1. General Introduction

1.1. Steroids

Steroids are, by definition, a large group of organic molecules including cholesterol, cholesterol derivatives, sex hormones, and drugs (Hanson, 2007; McDonnell et al., 1994). The basic structure of all steroids is the steran backbone. It consists of four fused rings, denoted A, B, C and D. Rings A, B and C are 6-membered naphthenes, ring D is a 5-membered naphthene (Shimizu, 1994). The contacts between A/B, B/C and C/D are in *trans-trans-trans* configuration. Otherwise, steroids vary significantly in their ring structures as well as in the functional groups attached to them. A variety of distinct steroids are found in humans, animals and other forms of life. Their functions are manifold. In the form of cholesterol they are significant constituents of plasma membranes of animal cells, as bile acids they are crucial for the digestion of hydrophobic nutrients, and in the form of steroid hormones they function as signaling molecules that regulate the development of organs, sex differentiation and maturation of animals, and various other physiological processes of organisms (Hellstroem and Lindstedt, 1964; McDonnell et al., 1994; Russell, 2003).

In mammalian cells, the biosynthesis process of steroids originates from mevalonate via the HMG-CoA reductase pathway. First, two molecules of acetyl-CoA condense to produce acetoacetyl-CoA. This reaction is under the control of acetoacetyl-CoA transferase or thiolase. Acetoacetyl-CoA is then further processed by various additional steps to dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) (Figure 1). DMAPP and IPP react together to form geranyl pyrophosphate (GPP). Then the GPP molecule and an additional IPP condense head-to-tail with simultaneous pyrophosphate release to farnesyl pyrophosphate. Two farnesyl pyrophosphates condense head-to-head to form squalene with the simultaneous release of two pyrophosphates. Squalene is then further processed via cyclisation of the four rings to lanosterol (Figure 1). Lanosterol is then further processed to cholesterol which constitutes the starting compound of all other steroids, including the steroid hormones (Liang et al., 2007; Svechnikov et al., 2001).

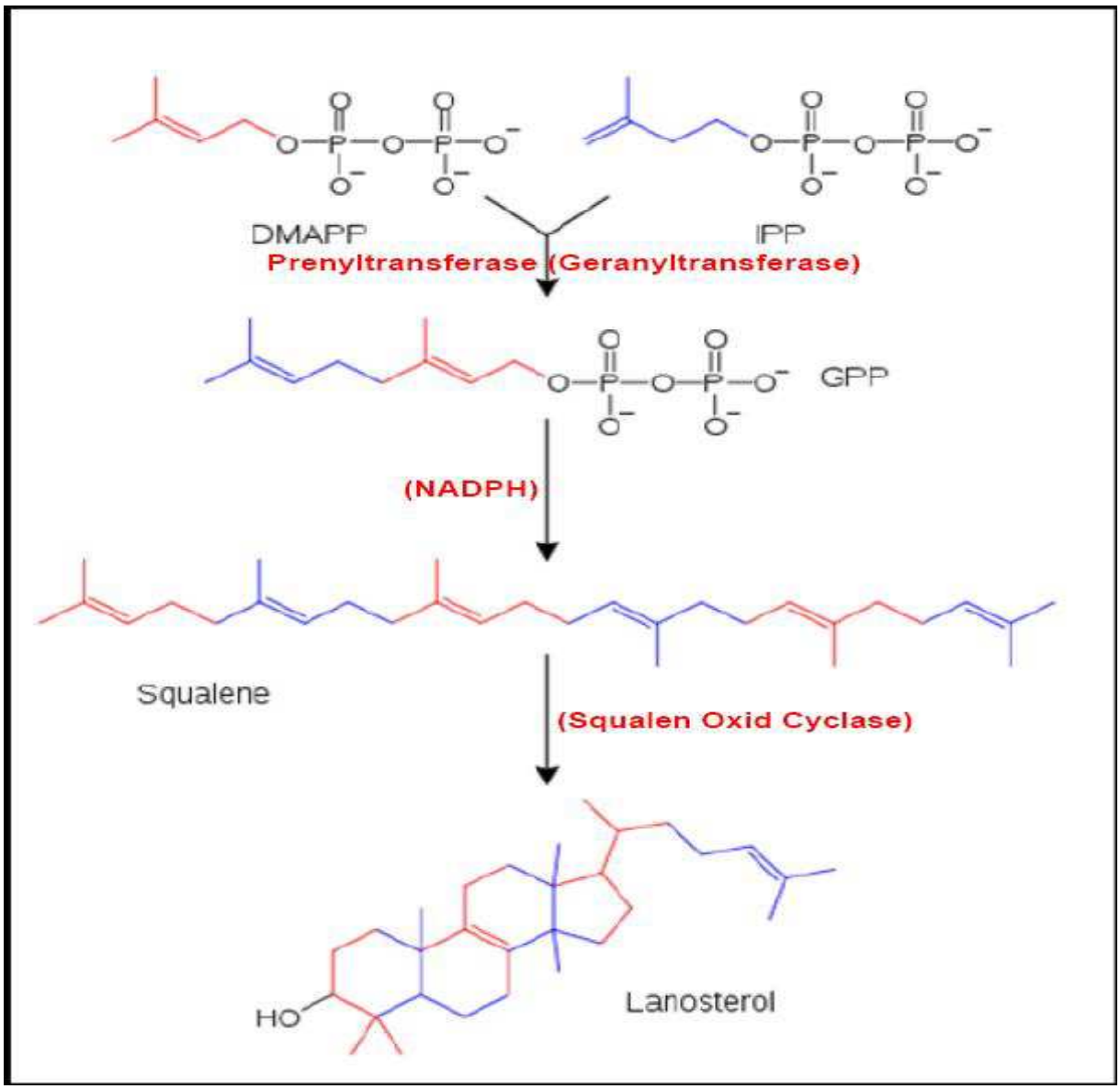

Figure 1: Simplified synopsis of the biosynthesis steps leading to the production of lanosterol, the source compound for cholesterol. (DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate)

1.2. Biosynthesis of Steroid Hormones

While cholesterol is synthesized in all mammalian cells, steroid hormone production is restricted to specific steroidogenic organs such as the gonads, the adrenal cortex, placenta and kidney (Vitamin D3, Calcitriol). The capacity, however, of these steroidogenic tissues to produce steroid hormones is not a perpetual process; it strongly depends on developmental stage, age or ovarian cycle (Arukwe et al., 2008; Connor et al., 2009; Maeyama et al., 1969; Valenti et al., 1997; Wiszniewska, 1998).

Most steroidogenic tissues are characterised through their ability to express the steroidogenic acute regulatory protein, commonly referred to as StAR (STARD1). StAR is a transport protein that regulates the rate-limiting step in the production of steroid hormones: the transfer of cholesterol into mitochondria (Arukwe et al., 2008; Budefeld et al., 2009). Post-translational modifications such as phosphorylation at serine 195, as well as mutations in its primary structure affect the activity of StAR. As a result, insufficient concentrations of important steroids may be produced and released, and adrenal hyperplasia constitutes a possible consequence of the defects that can result in death shortly after birth (Camats et al., 2014; Castillo et al., 2014; Khoury et al., 2004; Watari et al., 1997).

Cholesterol needs to be transported through the mitochondrial membrane by StAR primarily because of its lipophilic properties. Although the mechanism by which StAR accomplishes the translocation of cholesterol is not yet fully understood, the transfer of the steroid from the outer to the inner mitochondrial membrane is an absolute prerequisite for the biosynthesis of steroid hormones. The first decisive step for the biosynthesis of steroid hormones occurs at the inner mitochondrial membrane. The cytochrome P450scc (CYP11A1; scc= side chain cleavage), localized at the inner mitochondrial membrane, cleaves the cholesterol side chain to generate pregnenolone, the precursor of all steroid hormones (Arukwe, 2008; Arukwe et al., 2008; Miller, 1995). The cholesterol-pregnenolone conversion process is controlled by anterior pituitary tropic hormones such as ACTH and LH. Pregnenolone undergoes several modifications to form progesterone and other steroids, including testosterone (Figure 2). Pregnenolone can also be converted through 17α-hydroxylase (CYP17A1) to 17α-hydroxypregnenolone and then to dehydroepiandrosterone (DHEA), which constitutes the precursor of testosterone. It can also be converted mainly in the adrenal glands, the liver, and the small intestine by sulfotranferase (SULT2A1) to dehydroepiandrosterone sulphate (DHEAS) (Brand et al., 1998; Miller, 2004; New, 2003).

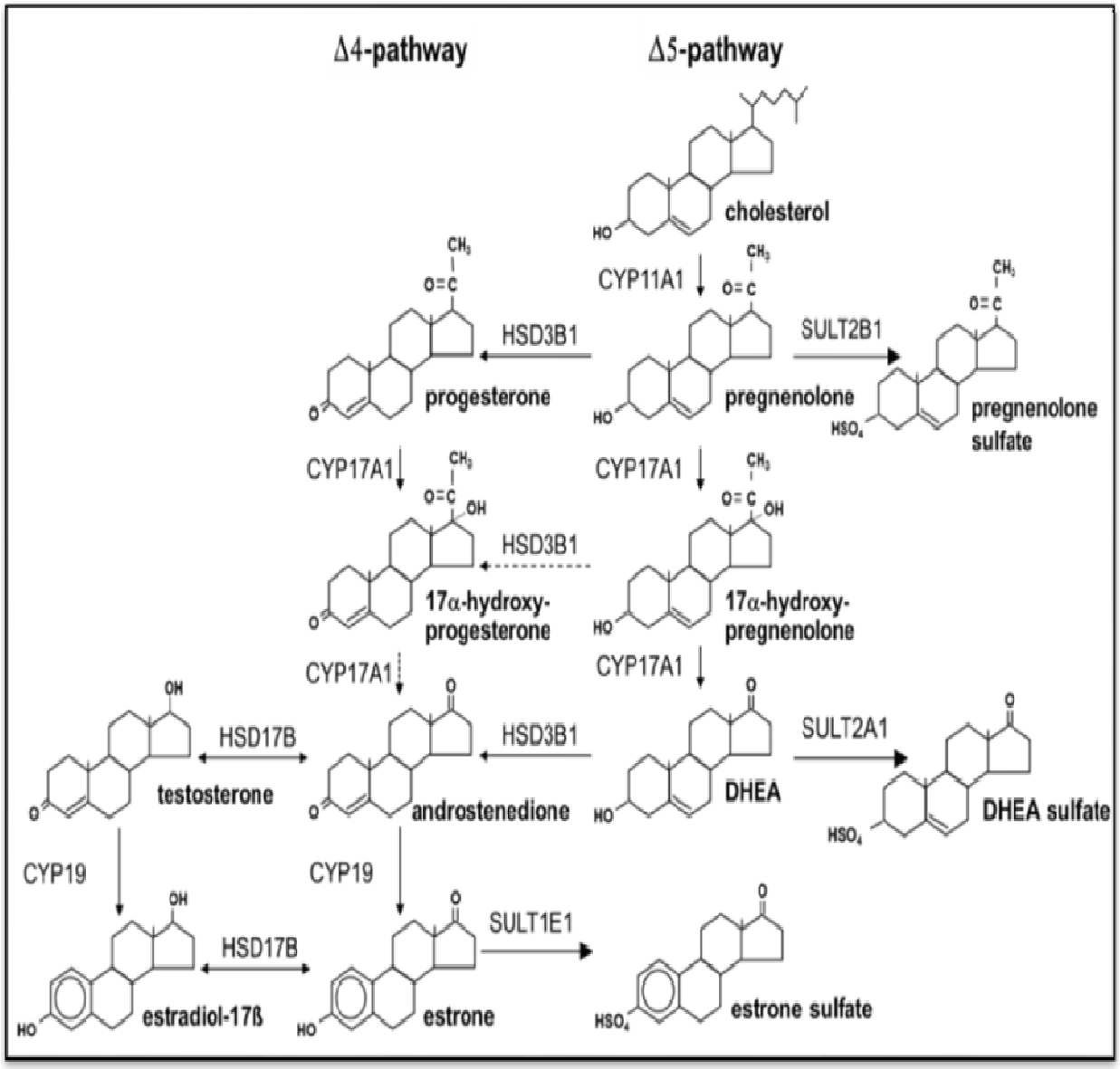

Figure 2: Conversion of cholesterol to pregnenolone, the precursor of all steroid hormones. The figure also displays the steps leading to the biosynthesis of testosterone and dehydroepiandrosterone sulfate (DHEAS).

1.3. Actions of Steroid Hormones

Hormones elicit their effects by interacting with specific receptors. Hydrophilic hormones such as peptide hormones are not capable of crossing the plasma membrane of cells. Therefore, they interact with receptors that are embedded in the plasma membrane. These receptors induce cytosolic signaling cascades associated with specific cellular or organ responses to the particular hormone. In contrast

hereto, lipophilic steroid hormones easily cross into the cytosol by diffusion through the plasma membrane. This hypothesis was confirmed in the 1960s, when the first cytosolic receptors for steroid hormones were identified. In the 1980s and 1990s the cloning of several of these receptors contributed to manifest the central dogma for steroid hormone action: steroid hormones exclusively signal through cytosolic/nuclear receptors. Since these pathways elicit changes in gene expression, the cellular responses to steroid hormone actions are referred to as *genomic effects*. The corresponding events that lead to the genomic effects are termed the *classical pathway of steroid hormones* (Valverde and Parker, 2002).

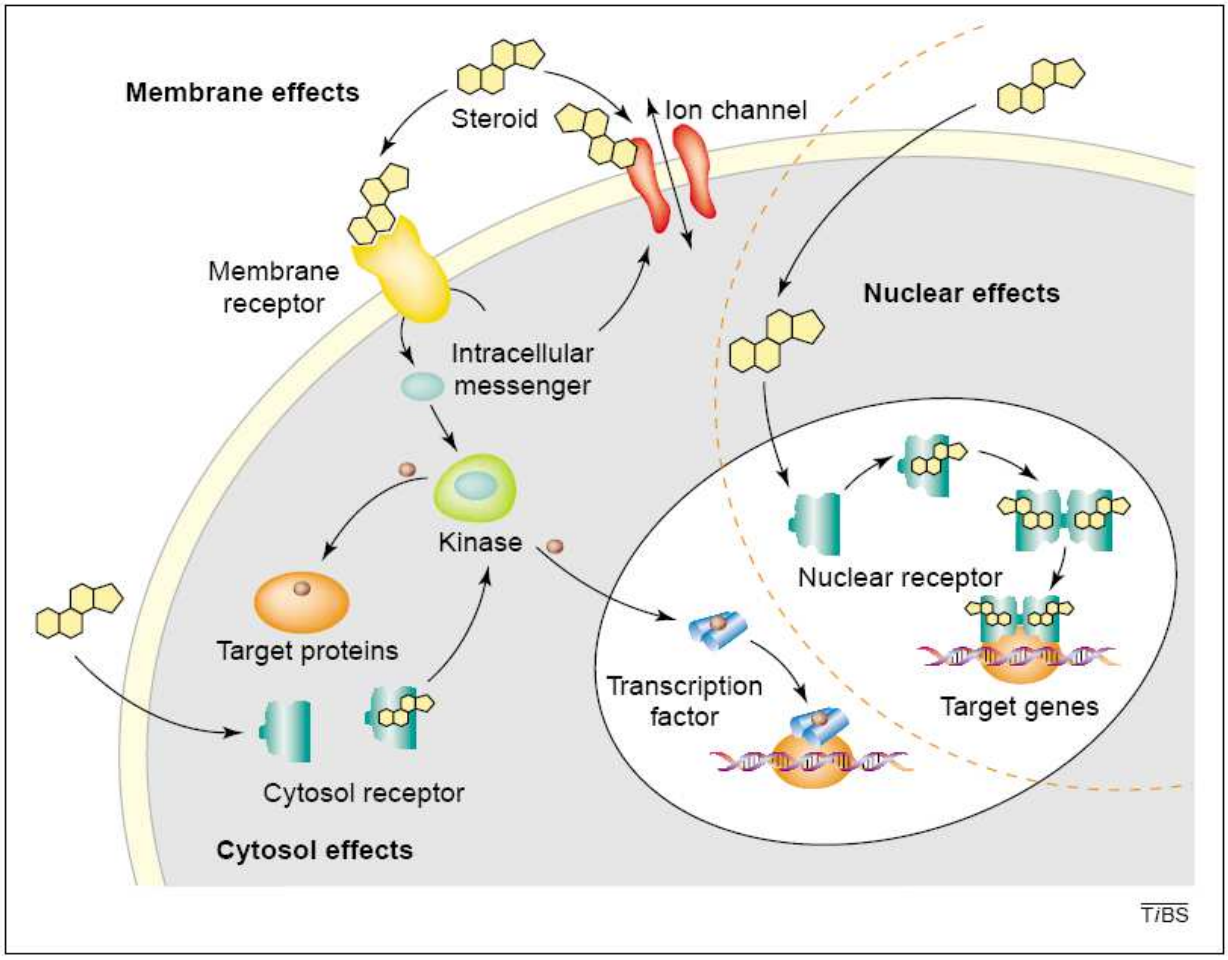

Figure 3: Distinct mechanisms of steroid actions at different cellular levels: Cytosolic membrane, cytosol, nuclear membrane, and nucleus (Valverde and Parker, 2002).

Genomic effects of steroid hormones are mediated through steroid hormone receptors (SHRs) that are basically ligand-activated transcription factors. In the absence of steroid hormone inactive SHRs linked to heat-shock protein remain in the

cytosol. After steroid hormones are transported through the plasma membrane by passive diffusion or through accelerated transport (Katsu et al., 2010; Kraus et al., 1995; Norman et al., 1992; Valverde and Parker, 2002), they bind to their specific SHRs. As this complex steroid hormone receptor changes its structure, the heat-shock protein separates, allowing the steroid hormone-receptor to dimerize. The steroid hormone receptor dimers penetrate the nuclear membrane, where by binding to specific HRE (hormone response element) they activate the target DNA and trigger the transcription process of the corresponding genes. Messenger ribonucleic acid (mRNA) is transcribed to be released later in the cytosol. The mRNA sequence is eventually translated to the corresponding protein (Daufeldt et al., 2006; Llopis et al., 2000; Vasudevan et al., 2005). This dogma, however, has not withstood further investigations. In an initial investigation Grazzini and co-workers identified a transcription-independent signaling pathway of the steroid hormone progesterone (Grazzini et al., 1998). Progesterone is essential for maintaining pregnancy in mammals, and it has an effect that is opposite to that of oxytocin, a nonapeptide that induces uterine contractions and may contribute to the onset of labour and parturition. Grazzini and coworkers were able to demonstrate that progesterone inhibits oxytocin signaling by binding to the membrane-bound oxytocin receptor. The oxytocin receptor belongs to the large class of membrane-bound receptors that relay their signals through guanine nucleotide binding (G) proteins to intracellular target proteins such as phospholipase C. Grazzini et al. found that progesterone inhibits two functional effects of oxytocin signaling: the production of the second messenger inositol 1,4,5-trisphosphate and an increase in the concentration of intracellular Ca^{2+}. By recording the changes in the Ca^{2+} concentration, they showed that inhibition takes place in less than a minute and is readily reversible.

It is now generally accepted that several steroid hormones interact not only with cytosolic/nuclear SHRs but also with membrane-integrated SHRs (Chen et al., 2005; Grazzini et al., 1998; Luconi et al., 2004). This type of receptor is probably present within membrane rafts. Interaction of steroid hormones with membrane-bound SHRs induces rapid signaling events that cannot be explained via the genomic mechanism of steroid action. Thus, erythrocytes which lack a nucleus were shown to respond rapidly -within seconds to minutes- to aldosterone, reducing the Na^{+} exchange

between the cells and the medium in vitro (Spach and Streeten, 1964). Similarly, estrogen and progesterone induce rapid signaling effects in spermatozoa, although their DNA is extremely compacted and not accessible to transcription factors (Baldi et al., 1995; Luconi et al., 2004; Rossato et al., 2005; Vicini et al., 2006). All of these rapidly induced signaling effects of steroid hormones are referred to as non-genomic effects in order to differentiate them from the genomic effects of steroid hormones described above. The corresponding signaling events that lead to the non-genomic effects comprise the non-classical pathway of steroid hormone action.

2. Aims of this thesis

Despite the general acceptance that steroid hormones can act through cytosolic/nuclear and membrane-bound receptors, a series of questions concerning the actions of specific steroid hormones and their metabolites remain unanswered. Membrane receptors for each steroid hormone have not yet been identified, even less so the signaling cascades that may be triggered through them. This applies for the androgen testosterone, which supposedly mediates all known signaling events solely through the cytosolic/nuclear androgen receptor (AR) that alters its position between cytosol and membrane. For other steroids like dehydroepiandrosterone sulfate (DHEAS) it is not clear whether they might be acting as hormones themselves or serve as pro-androgen that need to be converted to testosterone or other steroid hormones to exert their actions, or even if they simply represent waste products of steroid hormone metabolism.

Since both steroids mentioned are produced in testes, unveiling their mode of action might be of significant importance for male fertility and reproduction. For this reason the goal of the current investigation was:

2.1. Concerning DHEAS:

- To investigate the action of DHEAS on cells of the male reproductive system by focussing on a possible effects of its own in the generation of signaling cascades;
- to identify and describe the signaling cascades triggered;

- to identify the type of receptor that might be involved in the transmission of the signaling events.

2.2. Concerning testosterone:

- To analyse whether classical- and non-classical signaling of testosterone is mediated solely through the well-known cytosolic/nuclear androgen receptor;
- and -if not- to identify the type of receptor that might be involved in the non-classical signaling pathway.

3. Outcome of own investigations

3.1. Dehydroepiandrosterone sulfate mediates activation of transcription factors CREB and ATF-1 via a Gα11-coupled receptor in the spermatogenic cell line GC-2; *Mazen Shihan, Ulrike Kirch, Georgios Scheiner-Bobis; Biochimica et Biophysica Acta 1833 (2013) 3064–3075 (**see Attachment 1**).*

Dehydroepiandrosterone (DHEA) is mainly produced in the adrenal zona reticularis and is almost entirely converted by the enzyme sulfotransferase to dehydroepiandrosterone sulfate (DHEAS), which is then secreted into the serum (Burger, 2002). 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) (Chen et al., 2005). The levels of these steroids vary depending on gender and age. Their concentration in the body is the highest before the age of 29 years and declines afterwards steadily with increasing age (Abebe et al., 2003). Thus, DHEA concentration in the plasma of humans older than 80 years is almost 80% less when compared with the concentrations measured at ages below 29 (Birkenhager-Gillesse et al., 1994; Mazat et al., 2001; Salvini et al., 1992). DHEA and DHEAS are also produced in brain (Mensah-Nyagan et al., 1999), where their biological activity is considered to be neuroprotection (Maninger et al., 2009). While sulfated steroids have long been considered to be biologically inactive waste products of steroid hormone metabolism, the discovery of a cytosolic steroid

sulfatase (STS) prompted the new idea that the sulfates constitute a reservoir that upon desulfation can deliver precursors for steroid hormone synthesis (Dalla Valle et al., 2006). Thus, DHEAS has been viewed as a pro-androgen that, after being transported into cells, is desulfated by STS to DHEA and further converted into testosterone or other steroid hormones in order to exert its biological activity (Ebeling and Koivisto, 1994).

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 is not a prerequisite for certain actions and suggesting that caution should be used in interpreting the actions of either of the steroids. In support of this hypothesis, studies on rats show that STS inhibition can enhance neuronal functions that are also mediated by stimuli such as neurosteroids. This is caused by increased levels of DHEAS rather than of DHEA, which obviously enhance brain cholinergic function and lead to memory activation (Rhodes et al., 1997). In the same way, DHEAS was suggested to be involved in the development of tolerance to ethanol in mice (Barbosa and Morato, 2001; Barbosa and Morato, 2002; Barbosa and Morato, 2007). In addition, 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 (Krug et al., 2009; Ziegler et al., 2011). Similarly, DHEAS was shown to specifically stimulate growth factor-induced proliferation of bovine chromaffin cells in an age-dependent manner (Sicard et al., 2007). 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 (Sicard et al., 2007). Concerning their neuroprotective effects (Maninger et al., 2009), DHEA and DHEAS might be acting 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 (Kurata et al., 2004). In contrast, the neuroprotective effects of DHEAS against NMDA-induced cytotoxicity are most likely mediated through the Sig-1R receptor (Kurata et al., 2004).

An imbalance between levels of the sulfated steroid and its desulfated form has been shown to affect memory and harm the nervous system (Maurice et al., 2000; Maurice et al., 1999; Schumacher et al., 1997). This was also the case for low DHEAS serum levels in patients suffering from Alzheimer's disease (Nasman et al., 1996; Rasmuson et al., 1998). Based on these facts, a medical test has been developed to measure DHEA levels in plasma. It was found that DHEA and DHEAS levels in plasma are related to a very wide variety of diseases like breast cancer or tumours of the adrenal system (Barrett-Connor et al., 1990; Perrini et al., 2005; Schulz and Nyce, 1994; Tworoger et al., 2006), congenital adrenal hyperplasia (Bongiovanni, 1981; Young et al., 1994), memory loss (Taha et al., 2008; Traish et al., 2011), decreased bone and muscle mass, and gonad deformations and infertility (Grasso et al., 2015; Yonei, 2013). Thus, DHEA and DHEAS have been touted as a health-promoting food supplement which could be used in anti-aging and regenerative medicine (Bruckel, 2005; Hahner and Allolio, 2008; Rutkowski et al.; Von Bamberger, 2007). The physiological significance of DHEA versus DHEAS, however, is not yet sufficiently understood, and a connection has not yet been established between distinct effects of DHEA and DHEAS in different cell types and at their specific receptors (Widstrom and Dillon, 2004). Taking into consideration that DHEA and DHEAS are produced not only in the adrenal or brain but also in the gonads it is rather surprising that very little to nothing is known about the physiological significance of either steroid on biological processes associated with the reproductive system.

The investigations described in the publication summarized here clearly show that DHEAS induces specific effects on the spermatogenic cell line GC-2.

As demonstrated in Western blots and immunofluorescence experiments, DHEAS triggers in a time- and concentration-dependent manner the activation of a signaling cascade that includes the elements c-Src and Erk1/2 and results in the activation of the transcription factors CREB and ATF-1. The activation of this cascade is specific for DHEAS and does not require its conversion to DHEA or a different steroid. This conclusion is based on the fact that STS is not detectable in the GC-2 cells and is further supported by the fact that the STS-specific inhibitor irosustat ($C_{14}H_{15}NO_5S$),

also known as STX64, does not have any effect on the DHEAS-induced signaling cascade.

The DHEAS-induced signaling described in the publication resembles to a great extent the non-classical pathway of testosterone action. Thus, in order to rule out involvement of the classical pathway in the identified signaling cascade, the DHEAS effects were further assessed after silencing the expression of the cytosolic/nuclear androgen receptor (AR) at the mRNA and protein level by means of siRNA. The results obtained after the successful abrogation of AR expression demonstrate that a participation of the classical AR in the DHEAS-induced signaling cascade can be excluded. Neither Erk1/2 activation (investigated in western blots or by immunofluorescence) nor CREB or ATF-1 activation (demonstrated by immunofluorescence) were affected to any degree.

In contrast, silencing the expression of the G-protein Gα11 (equivalent to Gnα11 or Gq11) completely abolished the entire DHEAS-induced signaling cascade. Neither Erk1/2 nor CREB or ATF-1 was activated in immunofluorescence experiments nor Erk1/2 in western blots; these results suggest that DHEAS exerts its actions through its interaction with a membrane-bound G protein-coupled receptor (GPCR).

In summary, the investigation presented here calls into question the heretofore generally accepted idea of DHEAS being simply a pro-androgen that needs to be converted into testosterone or to another steroid hormone to be physiologically active. It also demonstrates for the first time that DHEAS acts as an autonomous 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. This signaling cascade involves a membrane-bound GPCR interacting with Gα11. The continuation of the work with the goal of identifying the membrane-bound DHEAS receptor and 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 new roles of DHEAS in male physiology and possibly also in female fertility and reproduction.

3.2. Non-classical testosterone signaling is mediated by a G-protein-coupled receptor interacting with Gnα11; *Mazen Shihan, Ahmed Bulldan and Georgios Scheiner-Bobis; Biochimica et Biophysica Acta 1843 (2014) 1172-1181 (**see Attachment 2**).*

As shown in Figure 2, testosterone is produced by various steroidogenic steps, involving mitochondria and endoplasmic reticulum (ER). The main location for testosterone biosynthesis is the Leydig cells of the male gonads. Smaller amounts are also produced in the adrenal glands of both sexes and to some extent also in ovaries. Nevertheless, testosterone produced in ovarian theca cells is almost completely converted to estrogens. Serum concentration of testosterone varies with age (Soeborg et al., 2014; Zirkin and Tenover, 2012). Its high concentration in males is critical for the development of the reproductive system including testis and prostate as well as for the manifestation of the secondary male characteristics (Bertolo et al.; Braux and Dufaure, 1983; Panchenko and Sergienko, 1983; Stahl et al., 1984; Wasson et al., 2000).

As described above, testosterone is known to act via two different pathways. In the classical (genomic) pathway, testosterone directly regulates gene transcription by binding to the cytosolic/nuclear androgen receptor (AR).

In the non-classical pathway, testosterone elicits 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 (Kato et al., 2000; Valverde and Parker, 2002). 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 (Freeman et al., 2005). These membrane-bound steroid receptors are often G-protein coupled receptors (GPCR) and therefore different from the nuclear SR (Filardo and Thomas, 2012; Lappano et al., 2013; Prossnitz et al., 2006). Nevertheless, the nature of the receptor involved in non-classical pathway of testosterone is a source of controversy. Whereas some investigators favour the exclusive participation of the well-characterized cytosolic/nuclear AR in both pathways (Walker, 2010), others propose a membrane-

bound AR, possibly from the family of G-protein-coupled receptors (GPCR), as mediator of several testosterone-induced effects (Dambaki et al., 2005; Estrada et al., 2003; Fu et al., 2012; Kampa et al., 2005; Kampa et al., 2002).

Non-classical action of testosterone on cells of the male reproductive system is essential for spermatogenesis and the maturation of spermatogonia to spermatozoa (Walker, 2010). CREB activation in Sertoli cells, which is required for the survival of spermatocytes and the production of mature spermatozoa (Scobey et al., 2001), 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 (Rahman and Christian, 2007; Walker, 2010; Walker, 2011). 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) (Almog and Naor, 2008; Li et al., 2009). In addition, Erk1/2 activation is an absolute requirement for the production of haploid spermatozoa (Di Agostino et al., 2004; Sette et al., 1999). The question still to be answered, however, is whether all of these effects are solely due to the interaction of testosterone with the classical AR 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.

The participation of a membrane-bound AR in the effects of testosterone would help to supplement or even revise some of the knowledge concerning the role of testosterone in male fertility and reproduction. It could also contribute to a better understanding of the effects of testosterone on cells outside the gonads or on prostate or testicular cancer cells.

For these reasons the current investigations addressed the role of the classical AR in testosterone-induced non-classical signaling in the spermatogenic cell line GC-2.

GC-2 cells respond to testosterone with activation (phosphorylation) of Erk1/2 and the transcription factors CREB and ATF-1. This response is consistent with the non-classical action of testosterone (Walker, 2010) and suggests 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 by restricting AR expression at the mRNA and protein level by means of siRNA. The

results summarized in Figs. 3-5 of the attached publication clearly show that silencing of the classical AR does not affect the induction of testosterone-induced signaling in GC-2 cells.

These data demonstrate that the classical AR does 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 the classical AR in Erk1/2 and CREB activation in Sertoli cells (Fix et al., 2004). In the absence of any alternative and satisfactory way to explain the discrepancy between the two investigations, one can only speculate at the current stage that the differences arise from the different cell types used.

Although a participation of the classical AR in the signaling events in GC-2 cells could be excluded, the nature of the additional AR involved in the signaling cascade induced by testosterone still remained obscured. Thus, a first attempt was undertaken to identify at least the receptor type to which the non-classical AR might belong. The fact that GPCR were suggested to be involved in the generation of testosterone-induced signaling in cell types such as myocytes (Estrada et al., 2003; Fu et al., 2012) or even Sertoli cells (Gorczynska and Handelsman, 1995; Loss et al., 2004), and based on our own results showing that 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 (Shihan et al., 2013), prompted us to investigate a possible involvement of Gnα11 in the actions of testosterone.

Silencing Gnα11 expression by means of siRNA at the mRNA and protein levels was found to have a great impact on the testosterone-induced signaling cascade in the GC-2 cells. Activation of Erk1/2, CREB and ATF-1 by testosterone, as demonstrated in immunofluorescence experiments and in western blots, was completely abrogated when Gnα11 expression was prevented, thus indicating not only the participation of this protein in the mediation of the non-classical testosterone pathway of the signaling cascade but also the existence of a membrane-bound GPCR as the non-classical AR.

Our data and conclusion are 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 (Filardo et al., 2002; Filardo and Thomas, 2012). 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 (Neuhaus et al., 2009). The identification of steroid hormone-specific GPCRs like 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, 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 involved in sexual maturation and effects of the non-classical testosterone pathway enabling rapid responses to transient stimuli.

3.3. Non-classical testosterone signaling in spermatogenic GC-2 cells is mediated through ZIP9 interacting with Gnα11; Mazen Shihan, Kai-Hui Chan, Lutz Konrad, Georgios Scheiner-Bobis*;* Cellular Signalling 27 (2015) 2077–2086 ***(see Attachment 3)****.*

A few months after the identification of Gnα11 as a mediator of the non-classical testosterone signaling (see Attachment 2), ZIP9, a Zn^{2+} transporter from the family of the ZRT, IRT-like transporting proteins (ZRT=zinc-regulated transporter; IRT=iron-regulated transporter) was identified by others as a testosterone-binding protein of plasma membranes, capable of inducing testosterone signaling. Thus binding of testosterone with high affinity (Kd=12.7 nM) to ZIP9 over-expressed in prostate or breast cancer cells leads to activation of Erk1/2, and to a testosterone-mediated Zn^{2+} accumulation and apoptotic cell death (Berg et al., 2014; Thomas et al., 2014). The signaling cascade is mediated through the interactions of ZIP9 with stimulatory Gsα proteins, as demonstrated by co-immunoprecipitation, testosterone-induced stimulation of [^{35}S]GTPγS binding to cell membranes from cells expressing

ZIP9, decreased specific [^{3}H]testosterone binding to membranes after treatment with excess GTPγS, and through the testostestrone-induced elevation of cellular cAMP levels (Berg et al., 2014; Thomas et al., 2014). Taking these and our findings into consideration, it was consequent to address whether the previously by us identified non-classical testosterone signaling in the spermatogenic cell line GC-2 (see Attachment 2) is also mediated by ZIP9 and if yes, whether ZIP9, in order to generate the signaling cascade, is interacting with Gnα11.

In the non-classical action of testosterone, activation of the Src/Ras/Raf/Erk1/2 signaling cascade leads to the activation of the transcription factor CREB. We therefore addressed by immunofluorescence and western-blotting a possible testosterone-induced activation of Erk1/2, CREB, and ATF-1 in GC-2 cells in the presence or absence of ZIP9, Gnα11 or classical androgen receptor (AR).

For the immunofluorescence analysis, control cells and cells that had been treated with negative-control siRNA (nc-siRNA), ZIP9-specific siRNA (ZIP9-siRNA), Gnα11-specific siRNA (Gnα11–siRNA), or AR-specific siRNA (AR-siRNA) were incubated with either 0 or 1 nM testosterone for 30 min and then subjected to fixation and immunostaining procedure. Phosphorylated (activated) forms of Erk1/2, CREB, or ATF-1 were detected by using appropriate primary antibodies and subsequent incubation with secondary antibody labeled with Fluorescein isothiocyanate (FITC).

In control cells and in cells treated with nc-siRNA 1 nM testosterone caused a significant stimulation of Erk1/2, CREB, or ATF-1. Similarly, a highly significant activation of these proteins by testosterone was also obtained when cells were treated with AR-siRNA to silence AR expression, indicating that the classical AR receptor is not the mediator of these signaling effects.

When, however, ZIP9 or Gnα11 expression was suppressed by the corresponding siRNAs the testosterone-induced activation of Erk1/2, CREB, or ATF-1, was completely obliterated, thus indicating the importance of both ZIP9 and Gnα11 for the non-classical testosterone signaling pathway.

Since immunofluorescence photomicrographs can only address cells or proteins that are within the optical field of the microscope, western blots were carried out to obtain a representative average by measuring the testosterone action on all cells.

Testosterone effects on GC-2 cells treated with nc-siRNA were compared with its effects on cells treated with ZIP9-siRNA, Gnα11-siRNA, or AR-siRNA. In cells treated with nc-siRNA or AR-siRNA, 1 nM testosterone stimulated within 30 min phosphorylation of Erk1/2, CREB and ATF-1. Treatment of GC-2 cells with ZIP9-siRNA, however, completely impaired the ability of testosterone to induce activation of Erk1/2, CREB or ATF-1. Expression of total Erk1/2 or total actin was unaffected by the treatment, indicating that the loss of p-Erk1/2, p-CREB or p-ATF-1 in ZIP9-siRNA treated cells was not due to an overall reduction in protein expression

Inhibition of Gnα11 expression had effects on testosterone signaling that were similar to those produced by inhibition of ZIP9 expression. Whereas treatment of cells with nc-siRNA did not impair the significant testosterone-induced stimulation of Erk1/2, CREB or ATF-1, exposure of the GC-2 cells to Gnα11-siRNA completely blocked the phosphorylation of the kinase and of both transcription factors. Total Erk1/2 or total actin was not affected by the treatment.

All western-blot results were consistent with the results of the immunofluorescence experiments and indicate that the non-classical signaling pathway of testosterone is not triggered by the interaction of the steroid with the known cytosolic/nuclear AR but rather through its interactions with ZIP9 and Gnα11. Do these two proteins, however, interact with each other or are they involved in different testosterone-triggered pathways? This question was addressed by a rather new method termed *in situ* proximity ligation assay (PLA). In order to address possible interactions between ZIP9 and Gnα11, the two proteins were targeted with a rabbit IgG and a mouse IgG, respectively. When both primary antibodies were present, red fluorescent dots indicating neighboring ZIP9 and Gnα11 were seen in each of the cells, suggesting a direct interaction of the two proteins. The fact that not a single red dot was observed in any of the cells when the ZIP9 or Gnα11 expression was abrogated by means of siRNA underlines the specificity of the PLA assay and supports the idea of direct interaction of ZIP9 and Gnα11 proteins.

4. Discussion

The investigation presented here deals with the effects of two steroid compounds that are both related to male fertility. As defined under "Goals of the Investigation", the first part of the work addresses a possible hormone-like action of DHEAS by investigating its capacity to generate signaling cascades in cells of the male reproductive system, by identifying and describing these signaling cascades, and, finally, by narrowing down the type of receptor with which the steroid interacts. The second part of the work investigates whether classical- and non-classical signaling pathways of testosterone are mediated through the same classical cytosolic/nuclear AR or whether a different type of receptor might be the source of the non-classical action of the steroid hormone.

The results obtained may indicate that DHEAS is indeed an autonomous hormone and not merely a pro-androgen that needs to be converted to a different steroid hormone to exert its action or even a waste product of steroid metabolism. Even at low concentrations it induces in the spermatogenic cell line GC-2 highly specific signaling cascades that, by including the elements c-Src, Erk1/2, CREB, and ATF-1, significantly overlap with the non-classical signaling pathway of testosterone. Nevertheless, prevention of the expression of the classical AR by means of siRNA does not affect the DHEAS-induced signaling cascade, indicating that the two apparently similar signaling pathways do not share the same receptor. Silencing the expression of the G-protein Gnα11 on the other hand results in the complete abrogation of all DHEAS-induced signals, consistent with a GPCR being the primary binding location for DHEAS.

The investigation of the testosterone-induced non-classical pathway unveils a similar signaling mechanism for this steroid hormone. Silencing the expression of the classical AR does not influence any of the signaling events induced by testosterone, indicating the presence of an additional testosterone receptor as the mediator of the activation of Erk1/2, CREB, or ATF-1. As in the case of DHEAS, however, the inhibition of Gnα11 expression by siRNA abrogates the testosterone-induced signaling cascade, suggesting that a membrane-bound testosterone receptor from the family of G protein-coupled receptors mediates the signaling.

G protein-coupled receptors (GPCRs) constitute one of the largest families of protein molecules; almost 800 different receptors in humans are known thus far (Maurice et al., 2011). They are all built according to the same blueprint: they all are membrane-embedded proteins with an extracellular N-terminus, an intracellular C-terminus, and seven transmembrane helices. Owing to the latter characteristic they are also referred to as heptahelical receptors. Despite this basic structural similarity, their ligands and actions are very diverse. GPCRs are involved in the recognition of messages as diverse as hormones, light, Ca^{2+}, odorants, small molecules including amino acid residues, nucleotides and peptides, or proteins. In short, more than 350 GPCRs are known to respond to different hormones, mating pheromones, and other stimuli. In contrast, the physiological significance and function of the bulk of GPCRs are still unknown (Herr, 2012).

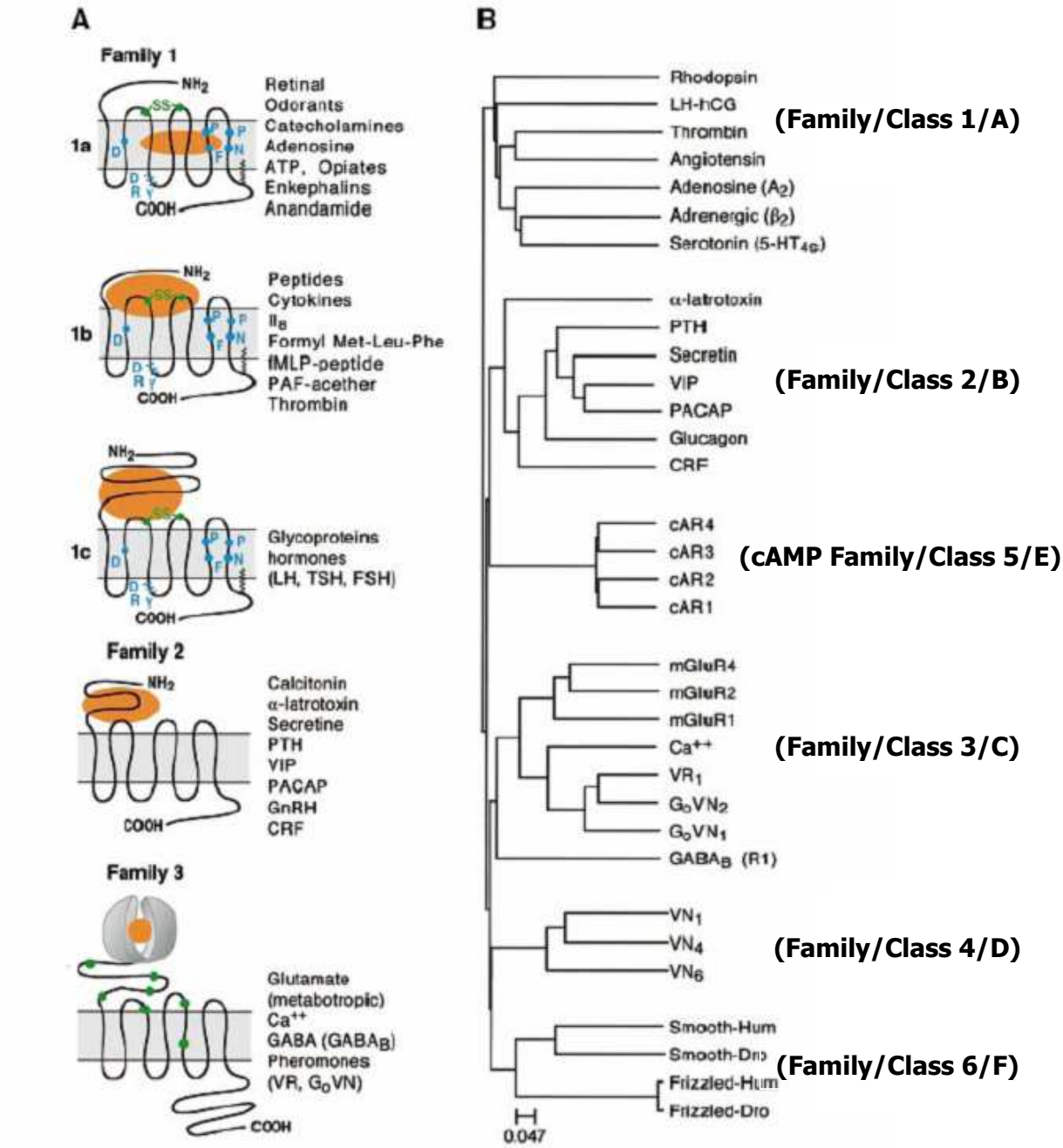

Figure 4: Classification and diversity of GPCRs. Class/Family: A (or 1) (rhodopsin-like), B (or 2) (secretin receptor family), C (or 3) (metabotropic glutamate/pheromone), D (or 4) (fungal mating pheromone receptors), E (or 5) (cyclic AMP receptors), F (or 6) (frizzled/smoothened). (**A**) The three main families and their effectors. (**B**) Family 4 comprises pheromone receptors (VNs). Family 6 includes the 'frizzled' and the 'smoothened' (Smo) receptors involved in embryonic development and in particular in cell polarity and segmentation. The cAMP receptors (Family 5) have been so far found only in the slime mold *Dictyostelium discoideum (Bockaert and Pin, 1999)*.

Based on their sequence similarity as well as on their predicted or known function, the great number of GPCRs has been divided into 6 families or classes (A—F or 1-6).

Family A (1), accounting for more than 85% of the identified GPCR genes, constitutes the largest family of GPCRs (Bockaert and Pin, 1999) (Fig. 4).

GPCR interact with G-proteins to induce various signaling cascades that control the activity of enzymes, of ion channels, vesicular transport and the overall physiology of cells, organs and organisms. There are several subtypes of Gα isoforms of G-proteins that together with Gβ and Gγ subunits form the functional heterotrimeric G-proteins (Table 1).

The heterotrimeric proteins are assembled from subunits taken from the G protein α (Gα), G protein β (Gβ) and G protein γ (Gγ) families.

Heterotrimeric G protein	Function
G protein α (Gα) subunits	
$G\alpha_s$	Stimulate adenylyl cyclase
$G\alpha_{olf}$	Stimulate adenylyl cyclase
$G\alpha_{i1}$	Inhibit adenylyl cyclase
$G\alpha_{i2}$	Inhibit adenylyl cyclase
$G\alpha_{i3}$	Inhibit adenylyl cyclase
$G\alpha_{o1}$	Inhibit adenylyl cyclase
$G\alpha_{o2}$	Inhibit adenylyl cyclase
$G\alpha_{t1}$	Stimulate cyclic GMP phosphodiesterase in rod photoreceptors
$G\alpha_{t2}$	Stimulate cyclic GMP phosphodiesterase in rod photoreceptors
Gz	Close K^+ channels. Inhibits exocytosis
$G\alpha_{gust}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_q$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{11}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{14}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{15}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{16}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{12}$	Stimulate RhoGEFs to activate Rho
$G\alpha_{13}$	Stimulate RhoGEFs to activate Rho
G protein β (Gβ) subunits; β1-β5	These β subunits combine with γ subunits to form βγ dimers that have a number of control functions
G protein γ (Gγ) subunits; γ1-γ11	These γ subunits combine with β subunits to form βγ dimers that have a number of control functions

Table 1: Heterotrimeric G-proteins and their function (Berridge, M.J. (2012) Cell Signalling Biology).

GPCRs, once activated by specific stimuli, interact with G-proteins to transmit specific signals through the plasma membrane by two different pathways:

A) The cyclic adenosine monophosphate (cAMP) pathway

B) The phosphatidylinositol pathway

A) In the cAMP pathway the activation of GPCR by a specific ligand results in alterations in the conformation of the receptor that is transmitted to a trimeric G protein complex. The stimulatory G protein alpha subunit Gα(s) is stimulated through the ligand-specific receptor. Once Gα(s) is activated, it exchanges GDP for GTP and separates itself from the β and γ subunits of the G protein complex. Adenylate cyclase is a plasma membrane-bound enzyme that is activated by the GTP-bound form of Gα(s). Activated adenylate cyclase catalyzes the conversion of ATP to cAMP which then acts as a second messenger. In mammals, the conversion of ATP to cAMP in the cytosol is mediated by members of Class-III AC/ADCY adenylate cyclase family. The increased concentrations of cAMP activate other components of cell signaling pathways like protein kinase A, which is also known as cAMP-dependent protein kinase (PKA). PKA phosphorylates other proteins or transcription factors such as CREB in the nucleus to initiate transcription. The effects of GPCRs coupled to Gα(s) are counteracted by the actions of a GPCRs coupled to Gα(i), and vice versa. Interaction of activated Gα(i) with adenylate cyclase leads to the inhibition of cytosolic cAMP formation, resulting in the inactivation of the PKA-mediated signaling events (Tomita et al., 2013).

B) In the phosphatidylinositol pathway, the ligand binds to its specific GPCR on the extracellular side of the plasma membrane and activates the Gαq isoform of G-proteins. In its inactive, GDP-bound form Gαq, being part of a trimeric G-protein, is associated with β and γ subunits. When activated, Gαq exchanges GDP for GTP, dissociates from the β and γ subunits, and activates phospholipase C (PLC). Active PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), which is located on the inner side of the plasma membrane, to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), which, in turn, phosphorylates other proteins, resulting in cellular responses. On the other hand, IP3 activates the IP3 receptor, a Ca^{2+} channel localized in the membranes of the smooth endoplasmic reticulum. This leads to the opening of the IP3 receptor/Ca^{2+} channel, leading to elevated intracellular Ca^{2+} concentrations. The increased concentrations of

Ca^{2+} amplify the PKC activation and thus regulate the Ca^{2+}/calmodulin kinase (CaMKII) pathway (Tomita et al., 2013).

Gnα11 by being related to Gαq is a G-protein that normally also acts through the phosphatidylinositol pathway (Tab. 1) and leads to the production of DAG and IP3 and to cytosolic [Ca^{2+}] elevation. In our experiments, however, activation of Gnα11 by either testosterone or DHEAS did not induce [Ca^{2+}] elevation, indicating the involvement of alternative pathways in the signaling events triggered by these steroids in the GC-2 cells. Since Gαq-activated PKC can also stimulate the Erk1/2

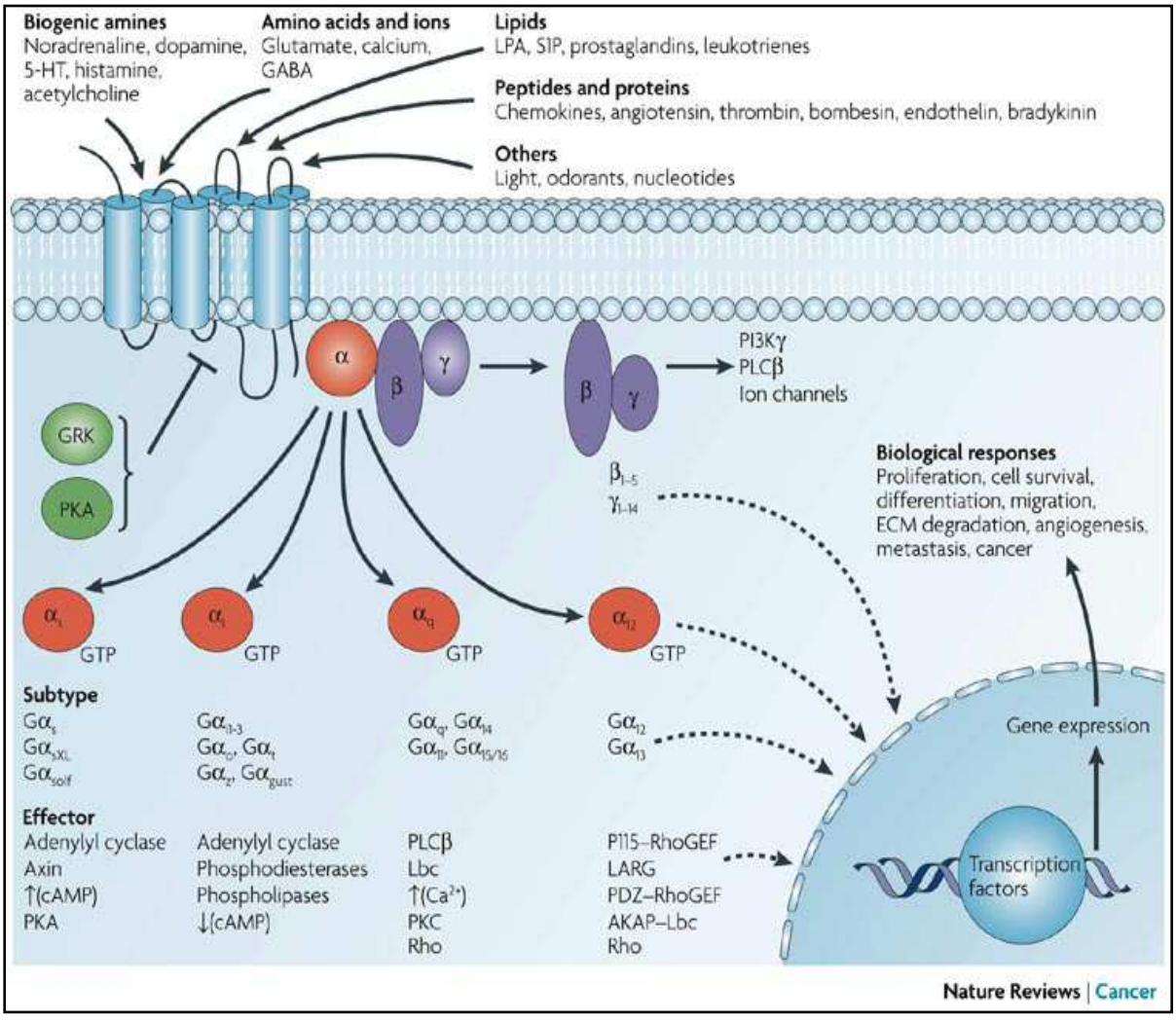

Figure 5: Heterotrimeric G-proteins composed of α, β, and γ subunits, and their different pathways through interacting with GPCRs induced by various stimuli (Dorsam and Gutkind, 2007).

pathway by directly phosphorylating c-Raf (Kolch et al., 1993; Schonwasser et al., 1998; Ueda et al., 1996), one might assume the involvement of this pathway in the signaling events detected here. As an alternative mechanism, the G$\beta\gamma$ subunits that

had been associated with the Gαq protein might also be involved in the activation of the Erk1/2 cascade, as seen in other investigations (Blaukat et al., 2000; Xie et al., 2000; Zhong et al., 2003). In any case, phosphorylation (activation) of Erk1/2 is a crucial event for the activation of different transcription factors like cAMP Response Element-Binding Protein (CREB), cAMP Response Element Modulator (CREM), and Activating Transcription Factor-1 (ATF-1), as was seen in the experiments involving either of the steroids DHEAS or testosterone. These transcription factors from the bZIP family interact with the transcriptional co-activator binding protein, bind to their specific DNA sites in the nucleus, and regulate gene transcription (Figure 5).

The investigations presented here demonstrate for the first time that DHEAS and testosterone trigger cellular signaling cascades that are generated through the involvement of the G-protein Gnα11. Based on current knowledge one has to assume the involvement of membrane-bound GPCRs. The identification of either of the receptors might be of physiological significance. Drugs that act through GPCRs are broadly used as therapeutics to treat a great number of human diseases as diverse as pain, hypertension, cognitive dysfunction, peptic ulcers, rhinitis, or asthma (Wise et al., 2004). Of the approximately 500 clinically marketed drugs, more than 30% act as modulators of GPCR function. At the same time they account for approximately 9% of total pharmaceutical sales. Thus, in terms of drug discovery GPCRs are the most important of all protein classes (Drews, 2000; Wise et al., 2004). GPCR-interacting drugs mediate their activity through approximately 30 well-characterized GPCRs. The human genome sequencing project has helped to identify approximately 720 genes that belong to the GPCR superfamily (Im, 2013; Wise et al., 2004). Roughly half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so-called orphan GPCRs with unknown ligand or function (Wise et al., 2004). The DHEAS and testosterone receptors are probably localized within this group of orphan receptors. Their deorphanization and the analysis and identification of the signaling cascades that are specifically activated by these receptors might help us to understand the role of either of the steroids in physiological and pathophysiological events.

Whereas our knowledge concerning the actions of DHEAS as a hormone is still rudimentary, the identification of the DHEAS receptor might bring enlightenment about the physiological roles of the sulfated hormone and aid in developing therapeutic concepts for the treatment of dysfunctions associated with male (or even female) reproduction and neuroprotection.

Although effects of testosterone are more widely studied than those of DHEAS, the molecular mechanism by which testosterone acts on cells, organs, or even organisms are not yet fully understood.

While the non-classical action of testosterone on cells of the male reproductive system is known to be essential for spermatogenesis (Walker, 2009; Walker, 2010) and Erk1/2 activation is critical for spermatogenesis (Almog and Naor, 2008; Li et al., 2009) as well as an absolute requirement for the production of haploid spermatozoa (Di Agostino et al., 2004; Sette et al., 1999), the AR receptor(s) involved in these signaling events is(are) not entirely identified yet. The same applies concerning the activation of cyclic AMP response element binding protein (CREB) in testicular Sertoli cells, which is required for the survival of spermatocytes and the production of mature spermatozoa (Scobey et al., 2001). While some investigations point towards the classical AR receptor as mediator (Rahman and Christian, 2007; Walker, 2010; Walker, 2011), our current results (Attachments 2 and 3) and the results of others (Berg et al., 2014; Thomas et al., 2014) showing that ZIP9 is involved in these signaling events not only contradict this assumption, they will probably also help to supplement our knowledge concerning the actions of testosterone on cells of the reproductive system and to provide a clearer picture of the involvement of this steroid hormone in the regulation of male fertility and reproduction.

In addition, the identification of ZIP9 as the mediator of the non-classical signaling pathway of testosterone might as well help to better understand many others of the manifold effects of this steroid hormone.

Testosterone-mediated non-genomic regulation of prostate cancer cell proliferation has been often associated with the classical cytosolic/nuclear AR (Heinlein and Chang, 2004; Liao et al., 2013; Wolff et al., 2012). Based on our current results (Attachments 2 and 3) and on the results of others (Berg et al., 2014; Thomas et al.,

2014) the situation might be more complex. Considering the fact that non-classical testosterone signaling in prostate cancer cells stimulates proliferative signals that occur within minutes and are mediated through Erk1/2 activation (Falkenstein et al., 2000; McCubrey et al., 2007; Peterziel et al., 1999; Roberts and Der, 2007), confirmation of ZIP9/Gnα11 interactions as an alternative route of androgen-induced signaling in prostate cells might help not only to reveal new actions of the steroid but also to pinpoint reasons for the resistance of various prostate tumors to anti-androgens and to develop new treatment methods aimed at the abrogation of all androgen-induced signaling, both classical and non-classical.

In addition, since non-classical testosterone signaling is also critical for cardiovascular, immune and musculoskeletal systems (Douglas et al., 2006; Rahman and Christian, 2007), the eventual confirmation of ZIP9 as the proposed membrane-bound AR and the analysis of its properties might help in understanding dysfunctions of these systems and possibly help to develop more effective concepts for therapy.

5. Summary

In addition to the so-called "classical" action of steroid hormones (SH) via intracellularly localized steroid hormone receptors (SHRs), SH can also induce signalling by so-called "non-classical" pathways, thought to be mediated through receptors on the plasma membrane. Neither the range of the signaling events nor the corresponding receptors involved in the fast signalling of the non-classical signaling pathways of SH have been characterized or even identified yet.

In order to complete current knowledge concerning the action of androgenic steroids and to further understand their actions and physiological relevance for cells of the male reproductive system it is crucial to characterize possible signaling events mediated through them and to identify the receptor types they are interacting with. Thus the focus of the investigation presented here was placed on the actions of two androgens that are abundant in the male gonads, dehydroepiandrosterone sulfate (DHEAS) and the testosterone.

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 close to nothing is known about its effects on cells of the reproductive system: neither DHEAS-specific signaling effects, nor their physiological significance or the type of receptor involved in the mediation of the signaling events have been assessed thus far. The work presented here demonstrates for the first time specific DHEAS-induced signaling events in a cell line derived from cells of the reproductive system. Thus, 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 ATF-1 and CREB. These actions are consistent with the non-classical signaling pathway of steroid hormones such as testosterone. Since DHEAS is considered a pro-androgen the question arises whether it has to be converted into testosterone in order to exert the effects identified. This assumption is clearly contradicted by the fact that steroid sulfatase mRNA was not detected in the GC-2 cells and by the clear demonstration that neither the presence of the steroid sulfatase inhibitor STX64 nor the abrogation of the androgen receptor expression by

siRNA prevented the DHEAS-induced activation of Erk1/2, ATF-1 and CREB. It therefore appears 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 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 might help to define new roles for DHEAS in the physiology of reproduction.

Like other steroid hormones, testosterone also mediates its effects by classical and non-classical pathways. Although the cytosolic/nuclear androgen receptor (AR), which serves as a ligand-activated transcription factor, is undoubtedly responsible for the classical, genomic actions of testosterone, the nature of the receptor involved in the non-classical pathway is a source of controversy. Next to the assumption that the membrane and cytosolic AR are identical, there is strong evidence that the AR of the membrane is a G-protein coupled receptor (GPCR). To evaluate either of the two possibilities we first searched for testosterone-induced signaling cascades in the spermatogenic cell line GC-2. We identified a testosterone-induced stimulation of Erk1/2, CREB and ATF-1 phosphorylation, equivalent to the already described non-classical action of testosterone. Silencing of AR expression by means of siRNA did not influence at all the androgen-induced activation of Erk1/2, CREB or ATF-1.

In contrast, suppression of the expression of the G-protein Gnα11 by siRNA abolished the testosterone-induced activation of Erk1/2, CREB and ATF-1, suggesting that the non-classical testosterone-induced signaling is not due to the interaction of the steroid with AR but rather with a plasma membrane receptor interacting with Gnα11.

This receptor is most likely ZIP9, a Zn^{2+} transporter from the family of the ZRT, IRT-like transporting proteins (ZRT=zinc-regulated transporter; IRT=iron-regulated transporter). Silencing its expression by means of siRNA abrogates all testosterone induced signaling such as Erk1/2, CREB or ATF-1 phosphorylation in the

spermatogenic cell line GC-2. Based on these findings and on the fact that ZIP9 and Gnα11 are most likely interacting proteins, as demonstrated by the close proximity assay, one can propose the involvement of ZIP9/Gnα11 in the mediation of the non-classical pathway of testosterone.

Taking into consideration the data obtained with both, DHEAS and testosterone, one might assume that non-classical signaling pathways of androgens -and maybe also of other steroid hormones- are in general mediated through GPCRs or other membrane proteins capable of interacting with G-proteins, and that Gnα11 might be a key component in the mediation of the androgen-induced signaling. Since non-classical androgen signaling is not only relevant for male fertility but also for the progression of male-specific cancers, as wells as for cardiovascular, immune and musculoskeletal systems confirmation of DHEAS interactions with GPCRs (to still be identified) or testosterone interactions with ZIP9/Gnα11 as an alternative route of androgen-induced signaling in further cell types and tissues might help not only to reveal new actions of the steroid but also to pinpoint reasons for the resistance of various prostate tumors to current anti-androgens and to develop new treatment methods aimed at all androgen-induced signaling, both classical and non-classical.

6. Zusammenfassung

Zusätzlich zu der so-genannten „klassischen" Wirkungsweise der Steroidhormone (SH) die durch intrazellulär-lokalisierten Steroidhormon-Rezeptoren (SHRs) vermittelt werden, induzieren SH auch „nicht-klassische" Signalkaskaden, die vermutlich über Membranrezeptoren vermittelt werden. Jedoch sind bislang weder der gesamte Umfang der nicht-klassischen Signalereignisse noch die hierfür zuständigen membran-assoziierten Rezeptoren umfassend charakterisiert oder gar identifiziert.

Um den jetzigen Kenntnisstand zur Wirkung und physiologische Relevanz von androgenen Steroiden für die Zellen des männlichen reproduktiven Systems zu erweitern ist erforderlich Signalereignisse, die durch diese induziert werden, zu charakterisieren und die Rezeptortypen zu identifizieren, die mit diesen interagieren. Daher fokussiert die hier präsentierte Arbeit auf die Wirkungsweise von

Dehydroepiandrosteron-Sulfat (DHEAS) und Testosteron, zwei androgenen Steroide, die in männlichen Gonaden reichlich vorkommen.

DHEAS ist ein zirkulierendes Steroid welches im Adrenalcortex, im Hirn und in den Gonaden produziert wird. Während eine Reihe von Untersuchungen dem Steroid neuroprotektive Wirkungen attestieren, ist überraschenderweise sehr wenig, ja fast nichts über seine Wirkung auf Zellen des reproduktiven Apparates bekannt: weder DHEAS-spezifische Signalkaskaden, noch ihre physiologische Signifikanz oder der Rezeptortyp, der die Signale vermittelt wurden bislang adressiert. Die hier vorgestellte Arbeit demonstriert zum ersten Mal spezifische, DHEAS-induzierte Signalereignisse in Zellen des reproduktiven Systems. So bewirkt DHEAS in der spermatogenen Zelllinie GC-2 eine zeit- und konzentrations-abhängige Phosphorylierung (Aktivierung) von c-Src und Erk1/2, sowie eine Aktivierung der Transcriptionsfaktoren CREB und ATF-1. Diese Signaleffekte entsprechen der „nicht-klassischen" Wirkung von Steroidhormonen, wie sie auch für das Testosteron beschrieben wurde. Da DHEAS als Proandrogen angesehen wird, ist es berechtigt zu fragen, ob es erst in Testosteron umgewandelt werden muss, um die Effekte zu triggern, die hier identifiziert wurden. Diese Vermutung muss jedoch verworfen werden, da weder die Anwesenheit des Steroidsulfatase-Inhibitors STX64, noch die Unterdrückung der Expression des Androgenrezeptors durch siRNA die DHEAS-induzierte Aktivierung von Erk1/2, CREB oder ATF-1 verhindern können. Es ist daher unwahrscheinlich, dass DHEAS zuerst im Zytosol in ein anderes Steroid umgewandelt werden muss, um die bereits erwähnten Kinasen und Transcriptionsfaktoren zu aktivieren. Es ist stattdessen sehr wahrscheinlich, dass die DHEAS-induzierte Signalkaskade durch die Interaktion des Steroids mit einem membran-gebundenen, G-Protein-gekoppelten Rezeptor (GPCR) vermittelt wird, da die Unterdrückung der Expression des G-Proteins Gnα11 zur Aufhebung der DHEAS-induzierten Stimulierung von Erk1/2, CREB oder ATF-1 führt. Die hier präsentierte Untersuchung zeigt zum ersten Mal eine hormon-ähnliche Wirkung von DHEAS auf eine spermatogene Zelllinie. Da DHEAS in männlichen und auch in weiblichen Reproduktionsorganen synthetisiert wird, könnten diese Befunde helfen neue Rollen des DHEAS in der Physiologie der Reproduktion zu definieren.

Wie andere Steroidhormone, so vermittelt auch Testosteron seine Effekte über klassische und nicht-klassische Signalwege. Obwohl der zytosolisch/nukleäre Androgenrezeptor (AR), der als ligand-aktivierter Transcriptionsfaktor wirkt, zweifelsohne für die klassischen, nicht-genomischen Effekte von Testosteron verantwortlich ist, wird über die Natur des Rezeptors, der in dem nicht-klassischen Signalweg von Testosteron involviert ist, kontrovers diskutiert. Neben der Vermutung, dass der membranständige und der zytosolisch/nukleäre AR identisch sind, deuten mehrere experimentelle Hinweise darauf hin, dass der membranassoziierte AR ein GPCR ist. Um diese zwei Möglichkeiten zu evaluieren, wurde zuerst nach Testosteron-induzierten Signalkaskaden in den spermatogenen Zellen GC-2 nachgeforscht. Die identifizierte Testosteron-induzierte Stimulierung von Erk1/2, CREB and ATF-1 entspricht der nicht-klassischen Aktion von Testosteron. Die Unterdrückung der AR-Expression mittels siRNA beeinflusst jedoch nicht im Geringsten die Androgen-induzierte Aktivierung von Erk1/2, CREB oder ATF-1.

Im Gegensatz hierzu, bewirkt die Unterdrückung der Expression des G-Proteins Gnα11 durch geeignete siRNA die vollständige Aufhebung der Testosteron-induzierten Aktivierung von Erk1/2, CREB und ATF-1, sodass man annehmen muss, dass der nicht-klassische Testosteron-induzierter Signalweg nicht durch den AR vermittelt wird, sondern durch einen Plasmamembran-gebundenen Rezeptor, der mit Gnα11 interagiert.

Dieser Rezeptor ist höchst wahrscheinlich ZIP9, ein Zn^{2+} Transporter aus der Familie der ZRT, IRT-like transporting proteins (ZRT=zinc-regulated transporter; IRT=iron-regulated transporter). Unterdrückung seiner Expression durch siRNA führt in der spermatogenen Zelllinie GC-2 zur Aufhebung aller untersuchten Testosteroneffekte, wie Erk1/2-, CREB- oder ATF-1-Aktivierung. Aufgrund dieser Befunde und der Tatsache, dass ZIP9 und Gnα11 untereinander interagieren, wie durch ein proximity ligation assay demonstriert wurde, kann man die Einbindung von ZIP9/Gnα11 in der Vermittlung der nicht-klassischen Signalweges von Testosteron annehmen.

Unter Berücksichtigung der Ergebnisse aus den Untersuchungen mit DHEAS und Testosteron, kann man vermuten, dass die nicht-klassischen Signalwege von Androgenen –und vielleicht auch die von anderen Steroidhormonen– generell durch

GPCR vermittelt werden und, dass dabei das Gnα11 eine Schlüsselkomponente der Weiterleitung der Androgen-induzierten Signalwege darstellt. Da die nicht-klassischen Signalwege der Androgene nicht nur für die männliche Fertilität sondern auch für die Progression von männerspezifischen Krebsarten und auch für Kardiovaskular-, das , Immun- und das Muskuloskeletalsystem von Relevanz sind, wird die Bestätigung von DHEAS-Interaktionen mit GPCR (die noch identifiziert werden müssen) oder von Testosteron-Interaktionen mit ZIP9/Gnα11 als Alternativroute des Androgen-induzierten Signalwegs auch in anderen Zelltypen und Geweben helfen nicht nur neue Wirkungsweisen der Steroide aufzudecken, sondern auch Gründe für die Resistenz verschiedener Prostata-Tumore gegenüber Antiandrogenen zu erfassen und neue Behandlungsmethoden zu entwickeln, die auf alle Androgen-induzierte Signale abzielt, klassische und nicht-klassische.

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II. Declaration/Deklaration

I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein.

I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on content of published or unpublished work of others, and all information that relates to verbal communications.

I have abided by the principle of good scientific conduct laid down in the charter of the Justus-Liebig University of Giessen in carrying out the investigations described in the dissertation.

Ich erkläre:

Ich habe die vorgelegte Thesis selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Thesis angegeben habe.

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Mazen Shihan

IV. The First Publication (*Attachment 1*)

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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

Mazen Shihan, Ulrike Kirch, Georgios Scheiner-Bobis *

Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Germany

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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-

* Corresponding author at: Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Str. 100, D-35392 Giessen, Germany. Tel.: +49 641 9938172; fax: +49 641 9938179.
E-mail address: Georgios.Scheiner-Bobis@vetmed.uni-giessen.de (G. Scheiner-Bobis).

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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

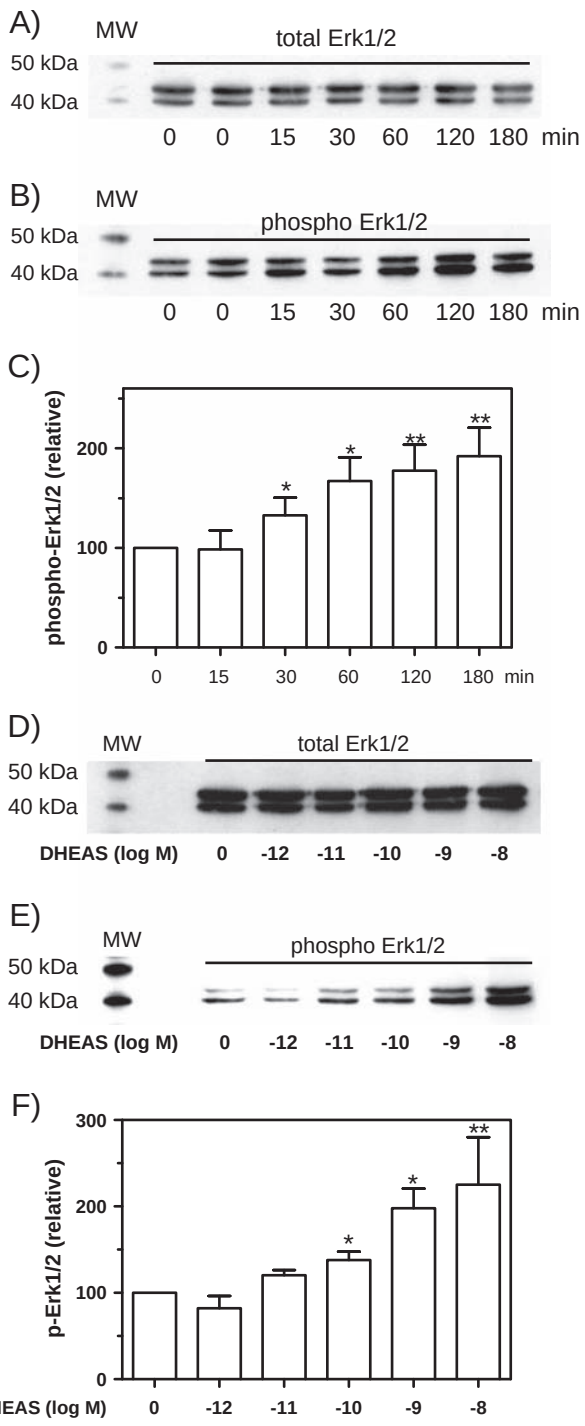

Fig. 1. Activation of Erk1/2 by DHEAS. Lysates of DHEAS-treated GC-2 cells were run on SDS-PAGE and subsequently probed in western blots. (A–C) Time-dependent activation of Erk1/2: While incubation with 1 nM DHEAS had no effect on the amount of total Erk1/2 (A), it stimulated its phosphorylation (B). 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.

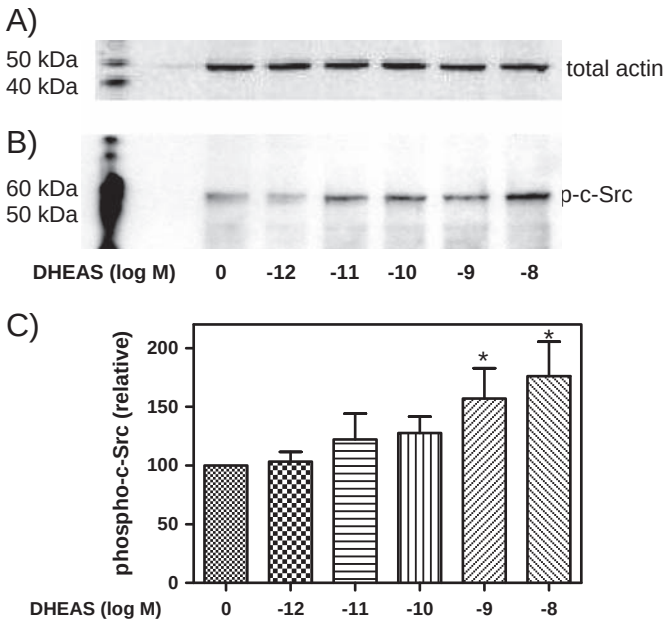

Fig. 2. Activation of c-Src by various concentrations of DHEAS. Conditions were the same as in Fig. 1D–E. Detection of total actin served as loading control (A). DHEAS stimulated c-Src phosphorylation (activation) at Tyr419 (B). Activation was significant at DHEAS concentrations ≥1 nM (B) ($n = 4–5$; 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).

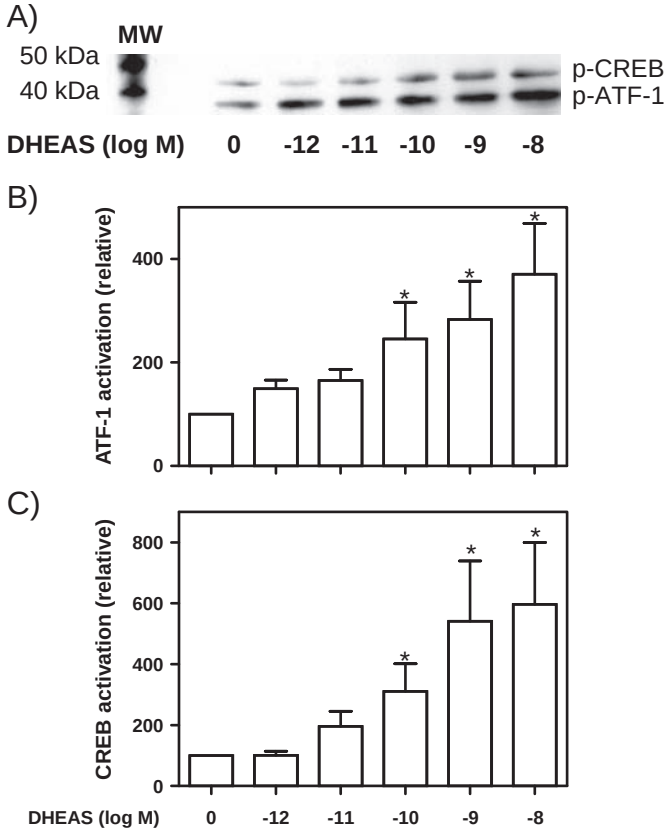

Fig. 3. DHEAS-induced activation of ATF-1 and CREB. Conditions were the same as for the experiments shown in Fig. 1D–E. For the western blot (A) an antibody against phospho-CREB was used that cross-reacts with phospho-ATF-1 (see "Materials and methods"). Activation of ATF-1 (B) or CREB (C) was significant 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′ACUCGAUCGCAUCAU UGCAUGCAAA3′ and 5′UUUGCAUGCAAUGAUGCGAUCGAGU3′; primer pair 2: 5′CCCAGAAGAUGACUGUALJCACACAU3′ and 5′AUGUGUGAUA CAGUCAUCUUCUGGG3′; and primer pair 3: 5′CCAGAUUCCUUUGCUGC CUUGUUAU3′ 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.

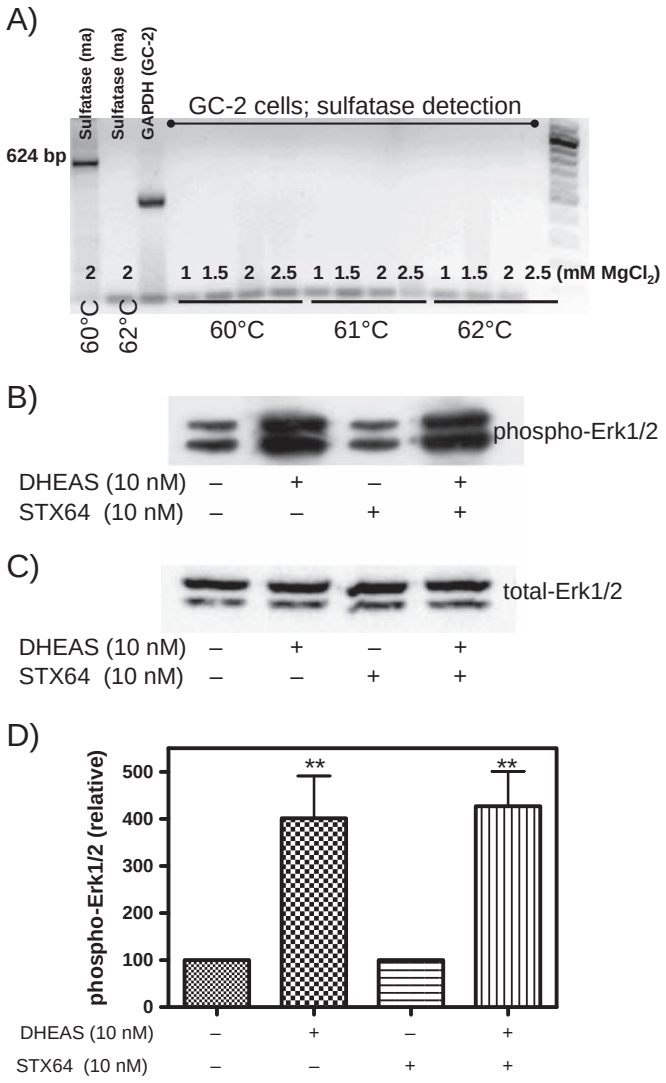

Fig. 4. Assessment of the involvement of steroid sulfatase in the DHEAS-induced signaling. (A) RT-PCR for the detection of steroid sulfatase-specific mRNA/cDNA. Steroid sulfatase-specific mRNA/cDNA was not detectable in GC-2 cell extracts at any annealing temperature and $MgCl_2$ concentration tested. 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′UUCUGGUA CACCAACUUGGtg′; 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

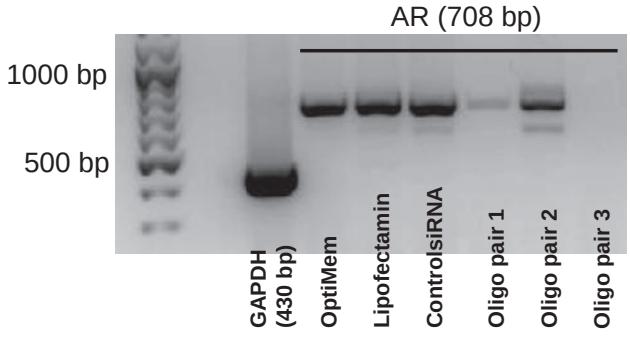

Fig. 5. Silencing expression of AR by means of siRNA. GC-2 cells were treated with three different nucleotide pairs of siRNA against AR according to the manufacturer's protocol. Control cells were treated with either Opti-MEM alone, Opti-MEM plus Lipofectamine, or both of the above plus negative control 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

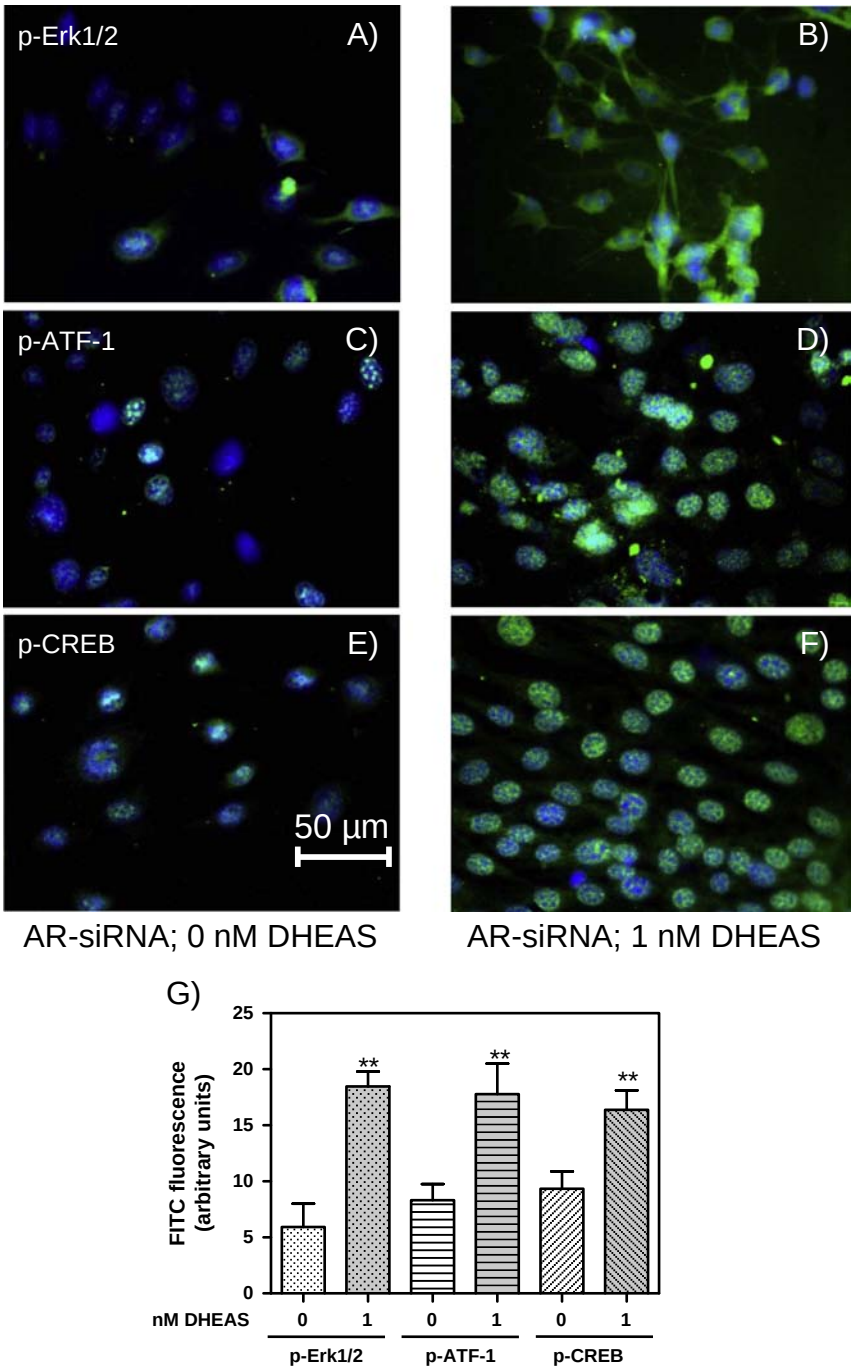

Fig. 6. Detection of phospho-Erk1/2, phospho-ATF-1 and phospho-CREB by immunofluorescence after silencing AR expression by siRNA. All cells shown were treated with oligo pair 3 to silence AR expression. Nuclei were stained with DAPI and with specific primary antibodies against phospho-Erk1/2, phospho-ATF-1 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.

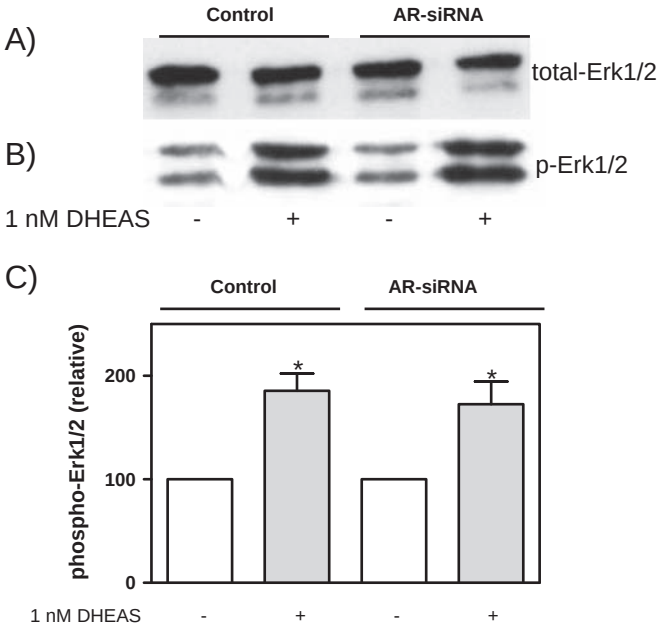

Fig. 7. Western blot analysis of phospho-Erk1/2 after silencing AR expression by siRNA. Cells were treated with control siRNA or with siRNA to silence AR expression. After 30 min of incubation in the presence or absence of 1 nM DHEAS, cell lysates were prepared and probed in a western blot as described 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).

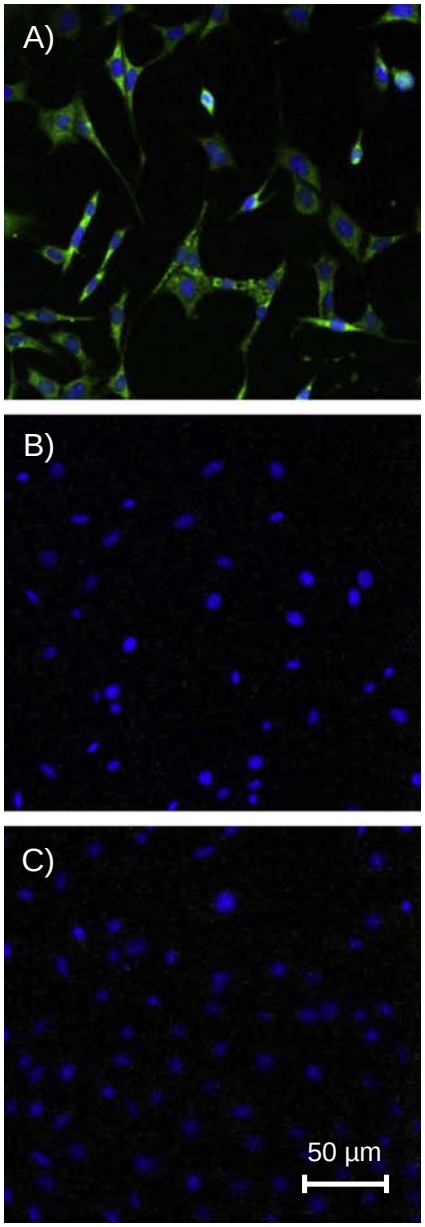

Fig. 8. Silencing AR protein expression by siRNA. Cells were fixed in methanol and incubated with a primary antibody against the AR and a fluorescent secondary antibody (rabbit anti-goat IgG-FITC green), as described under "Materials and methods". Nuclei are stained by DAPI, as described under "Materials 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

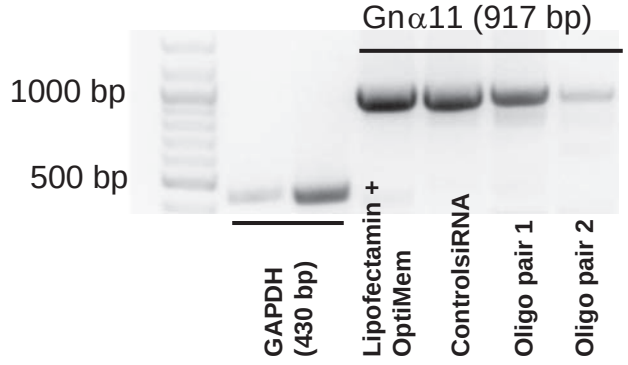

Fig. 9. Silencing expression of Gnα11-specific mRNA/cDNA by means of siRNA. Cells were incubated with 2 different oligonucleotide pairs (oligo pair 1 or 2) to silence Gnα11-specific mRNA expression. Isolation of mRNA and RT-PCR were carried out as described under "Materials and methods". Oligonucleotide 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

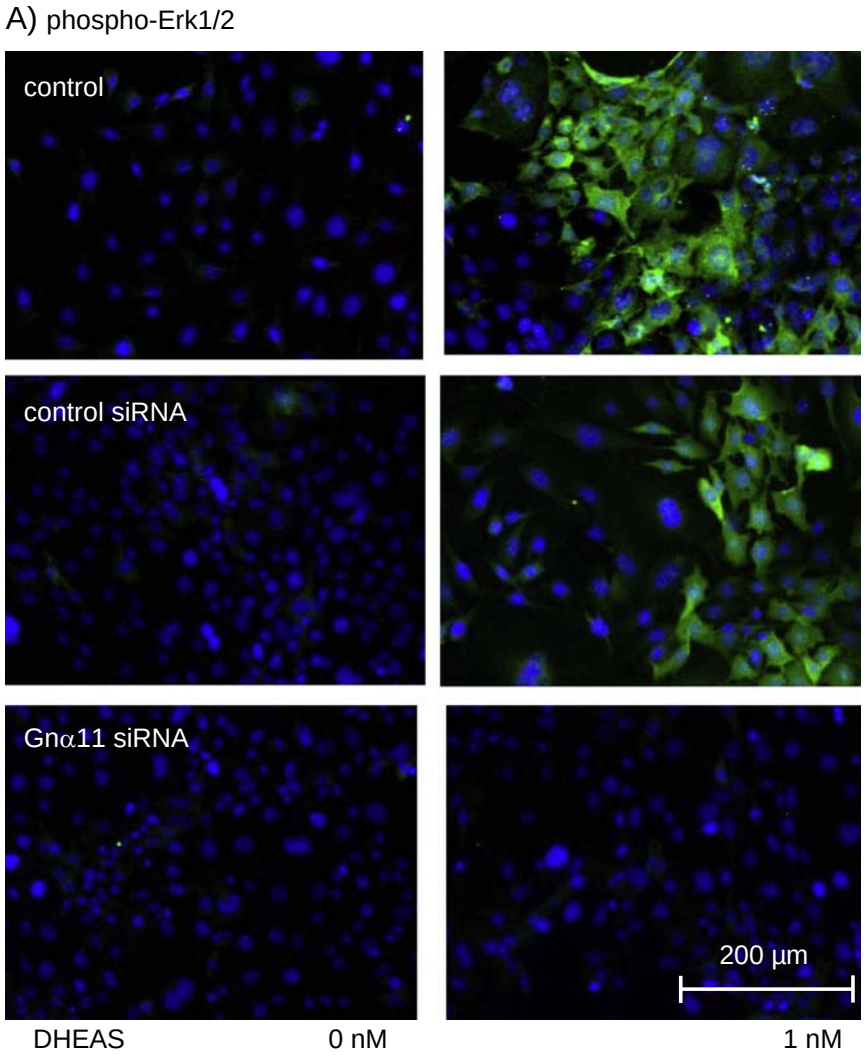

Fig. 10. Detection of phospho-Erk1/2, phospho-ATF-1 and phospho-CREB by immunofluorescence after silencing Gnα11 expression by siRNA. (A–C) Left columns: cells were treated with vehicle only (no DHEAS). Right columns: cells received 1 nM DHEAS. Upper rows: controls (untreated cells). Middle rows: controls 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; ** = $p \leq 0.01$).

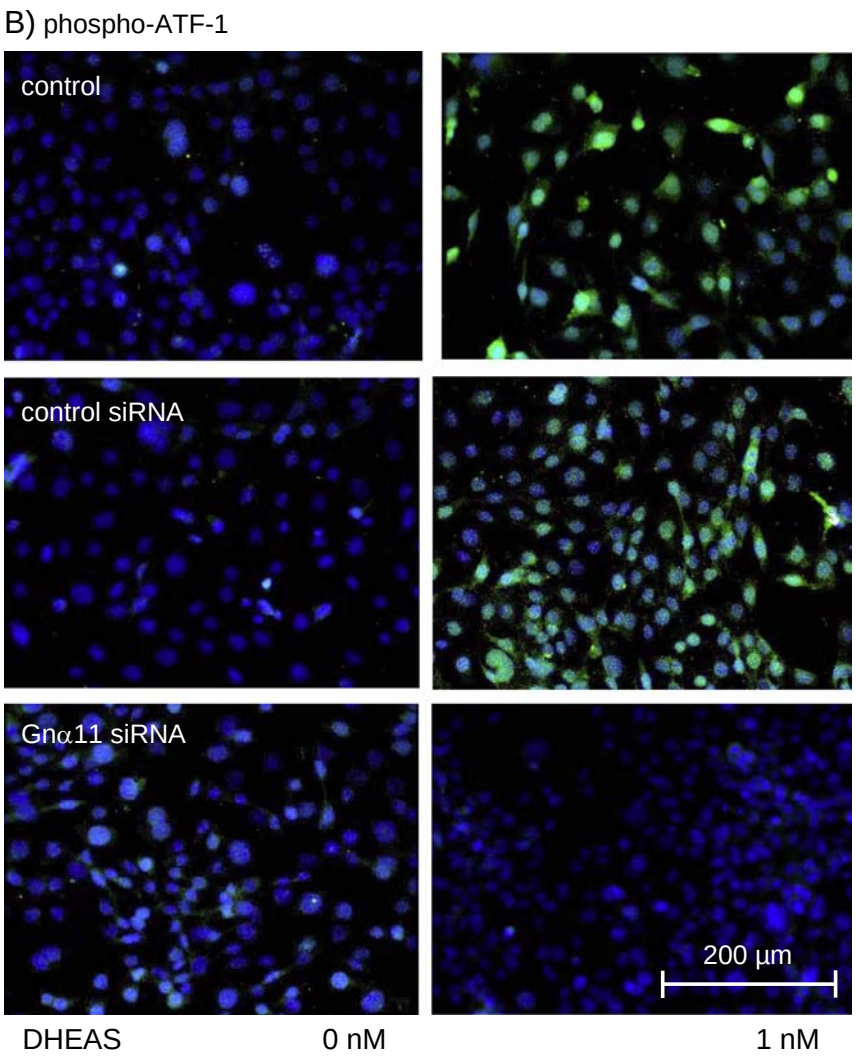

Fig 10 (*continued*).

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.

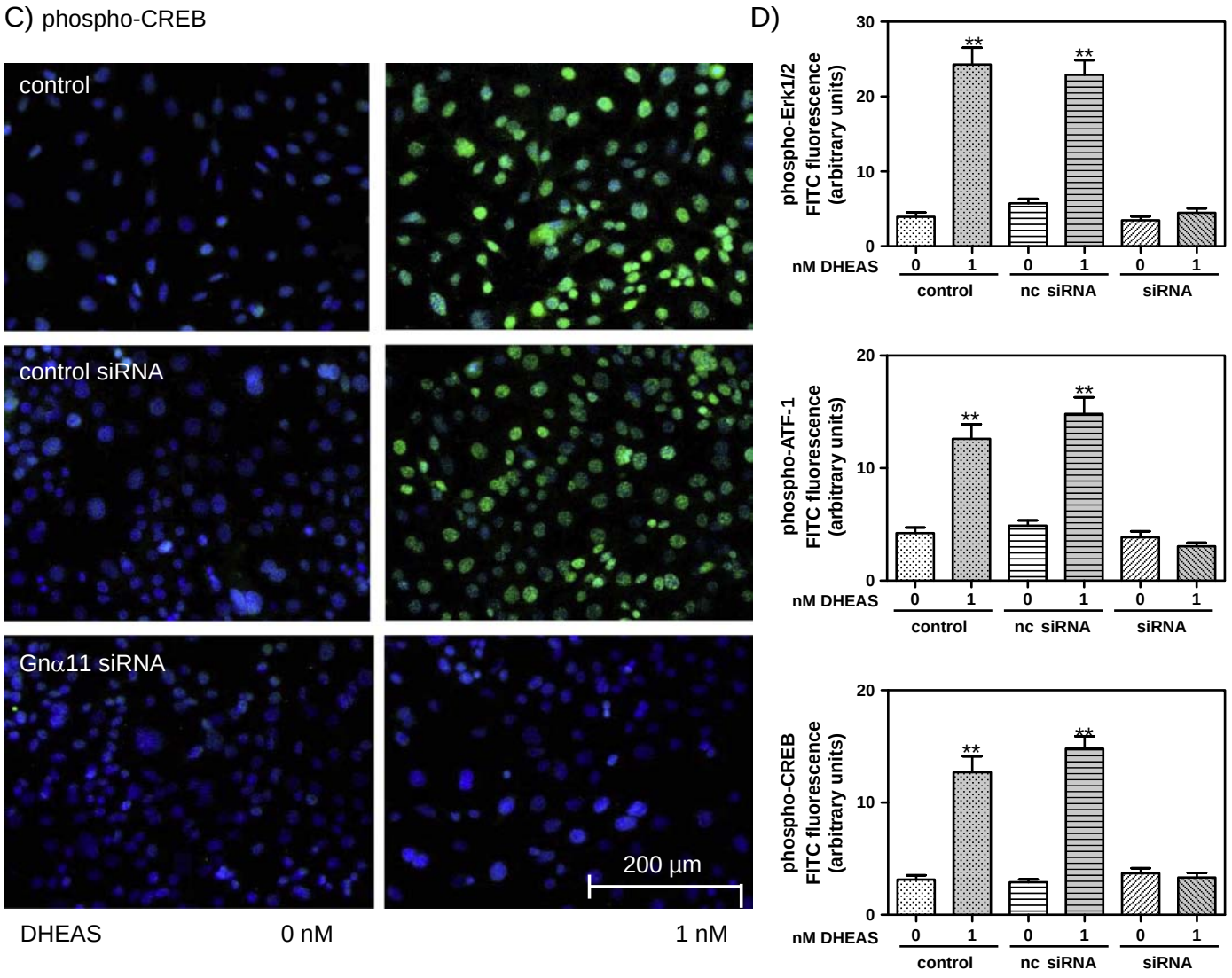

Fig 10 (*continued*).

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

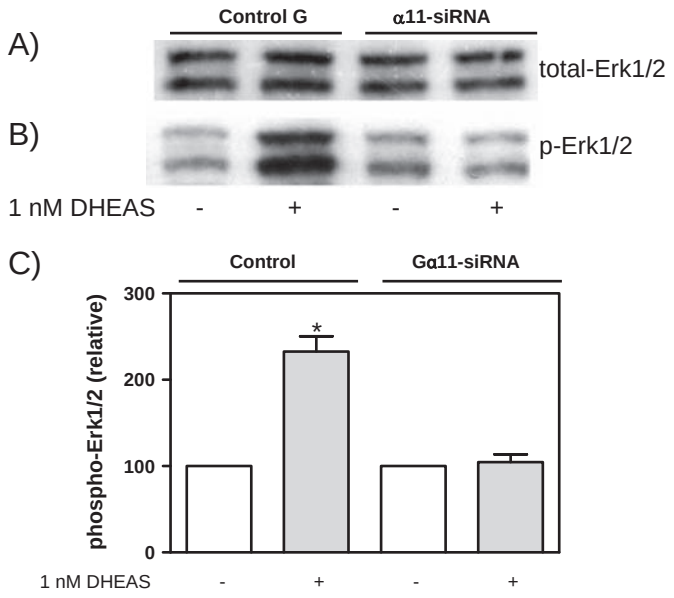

Fig. 11. Western blot analysis of phospho-Erk1/2 after silencing Gnα11 expression by siRNA. The preparation of cell lysates and the detection of proteins in western blots are described under "Materials and methods". (A) The amount of total Erk1/2 was not affected during the course of the incubation. (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.

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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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V. The Second Publication *(Attachment 2)*

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

Mazen Shihan, Ahmed Bulldan, Georgios Scheiner-Bobis *

Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Germany

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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

* Corresponding author at: Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Str. 100, D-35392 Giessen, Germany. Tel.: +49 641 9938172; fax: +49 641 9938179.
E-mail address: Georgios.Scheiner-Bobis@vetmed.uni-giessen.de (G. Scheiner-Bobis).

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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

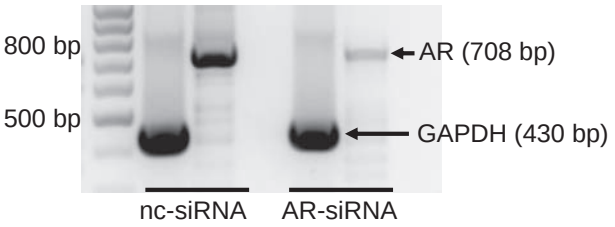

Fig. 1. Silencing expression of AR-specific mRNA using siRNA. GC-2 cells were treated with either AR-siRNA or negative-control siRNA (nc-siRNA). Total RNA was then isolated and subjected to RT-PCR to amplify AR-specific mRNA/cDNA fragments of 708 bp. Treatment of the cells with AR-siRNA abolished the 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.

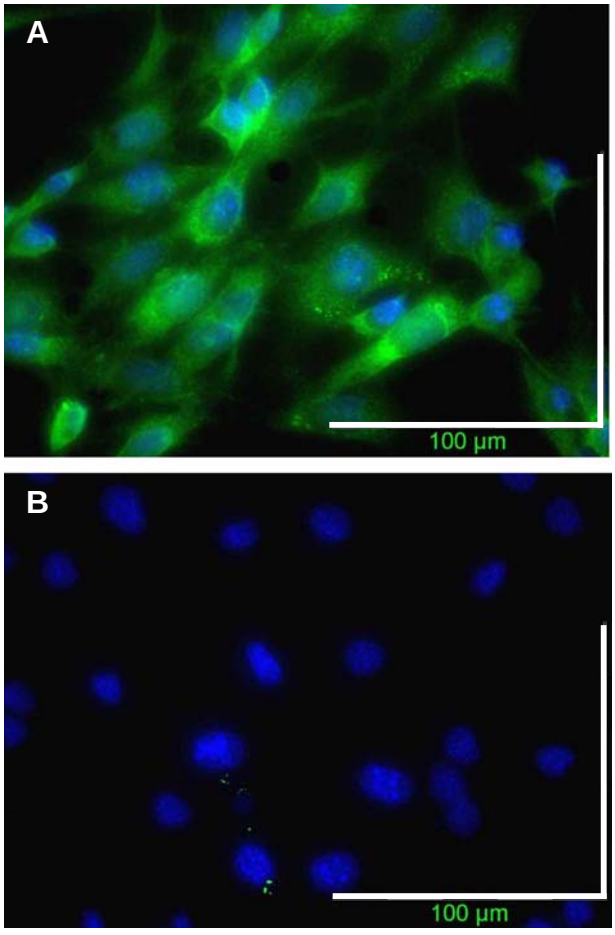

Fig. 2. Silencing AR protein expression by siRNA. Cells treated as described in Fig. 1 were fixed in methanol and incubated with a primary antibody against the AR and a fluorescent secondary antibody (rabbit anti-goat IgG-FITC green). Nuclei were stained with DAPI. (A) All cells treated with nc-siRNA 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

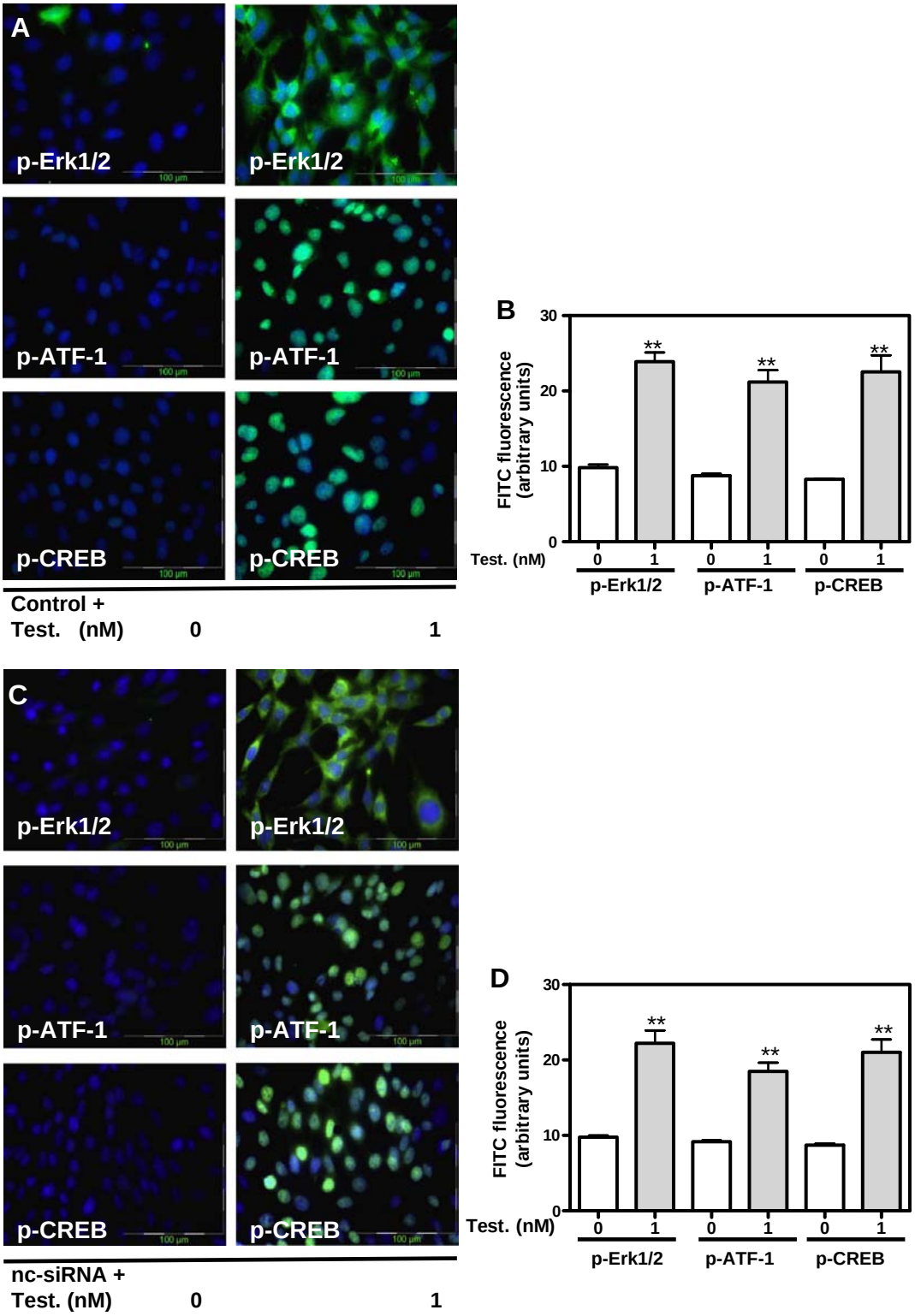

Fig. 3. Testosterone-induced activation of Erk1/2, ATF-1, and CREB in the presence or absence of AR detected by immunofluorescence. (A) GC2 cells that had not been treated with siRNA were incubated with testosterone and then fixed in methanol; nuclei were stained with DAPI. p-Erk1/2, p-ATF-1, or p-CREB 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; ** = $p \leq 0.01$.

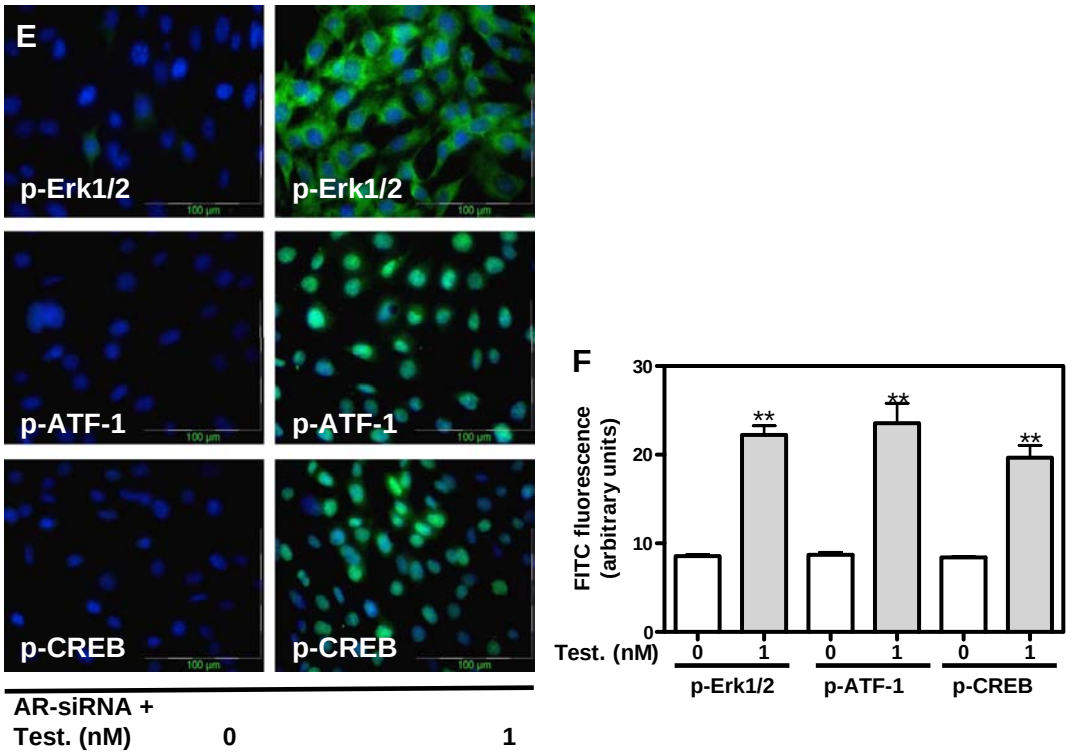

Fig. 3 (*continued*).

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-

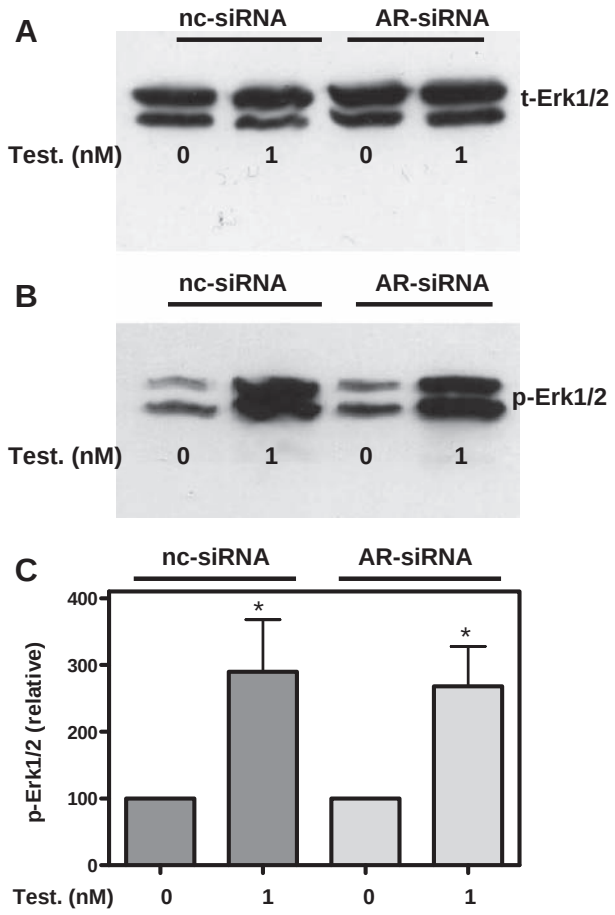

Fig. 4. Western blot analysis of p-Erk1/2 after silencing AR expression by siRNA. Cells were treated with either nc-siRNA or with AR-siRNA. After 30 min of incubation in the presence or absence of 1 nM testosterone, cell lysates were prepared and probed in western blots. (A) Incubation with 1 nM testosterone 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; * = $p \leq 0.05$).

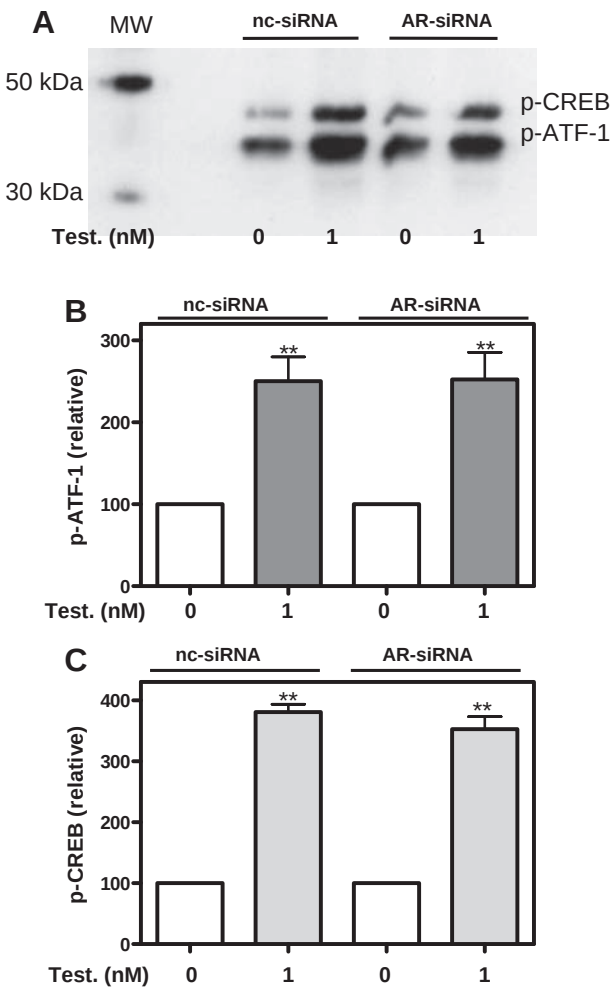

Fig. 5. Western blot analysis of p-ATF-1 and p-CREB after silencing AR expression by siRNA. Conditions are the same as in Fig. 4. (A) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA or AR-siRNA to the same extent. Analysis of pooled data like those shown in A ($n = 3$; 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

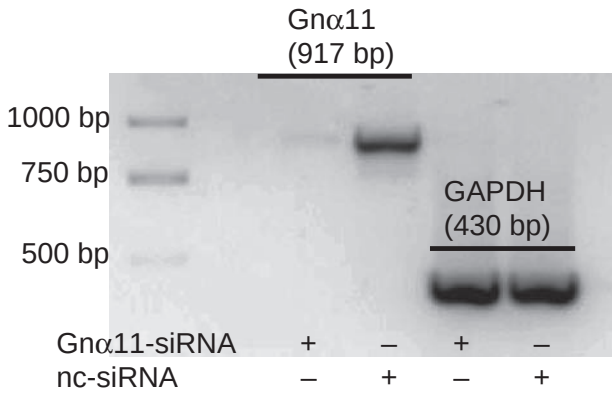

Fig. 6. Silencing expression of Gnα11-specific mRNA/cDNA by siRNA. Cells were incubated with nc-siRNA or Gnα11-siRNA to silence Gnα11-mRNA expression. Total RNA was then isolated and subjected to RT-PCR to amplify Gnα11-specific mRNA/cDNA fragments of 917 bp. While Gnα11-specific mRNA/cDNA was clearly 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).

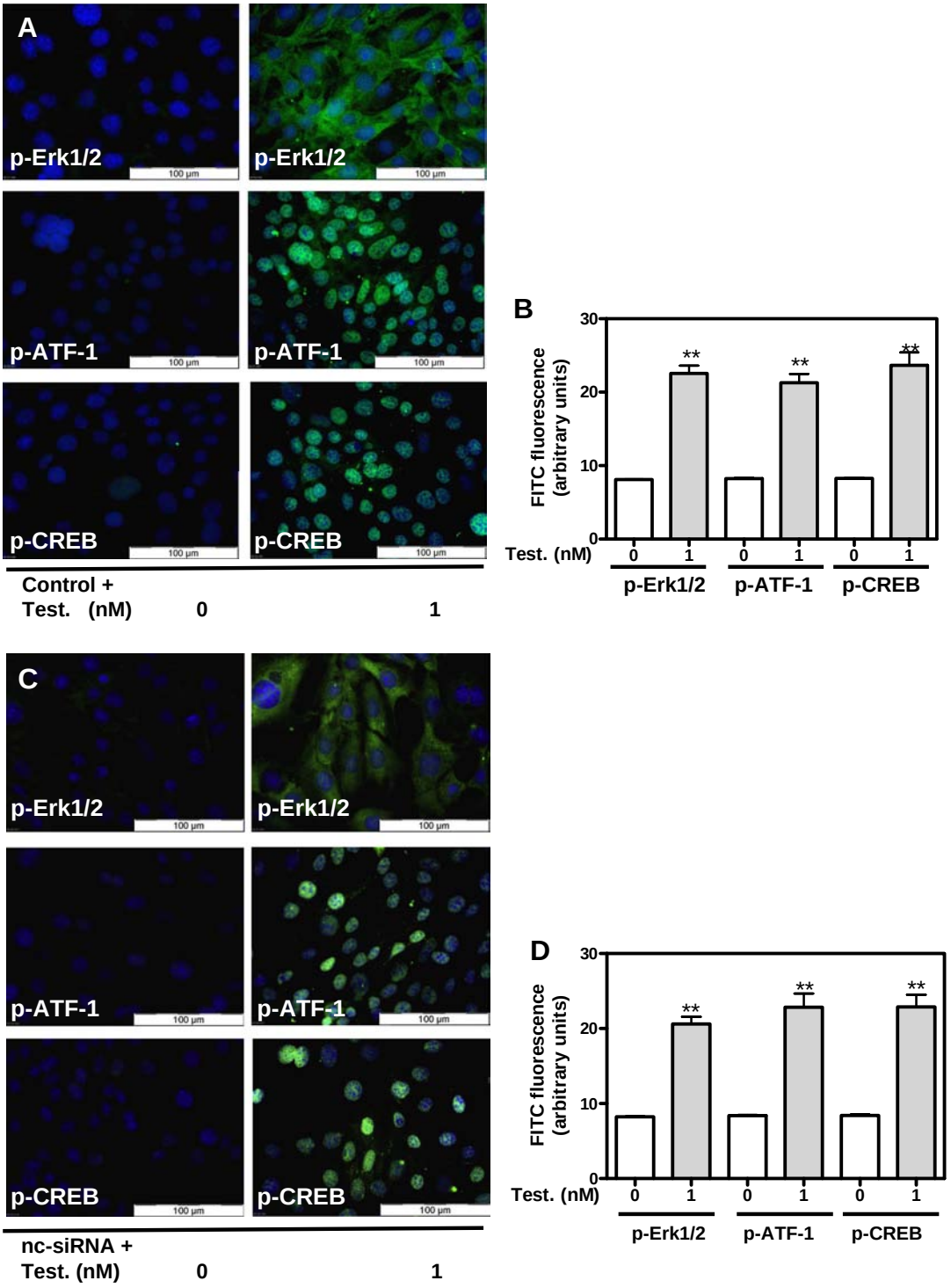

Fig. 7. Detection of testosterone-activated Erk1/2, ATF-1, and CREB by immunofluorescence after silencing Gnα11 expression by siRNA. (A) After treatment with 1 nM testosterone (right panels) almost every single cell in the optical field was fluorescent, indicating activation of Erk1/2, ATF-1, and CREB (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; ** = $p \leq 0.01$).

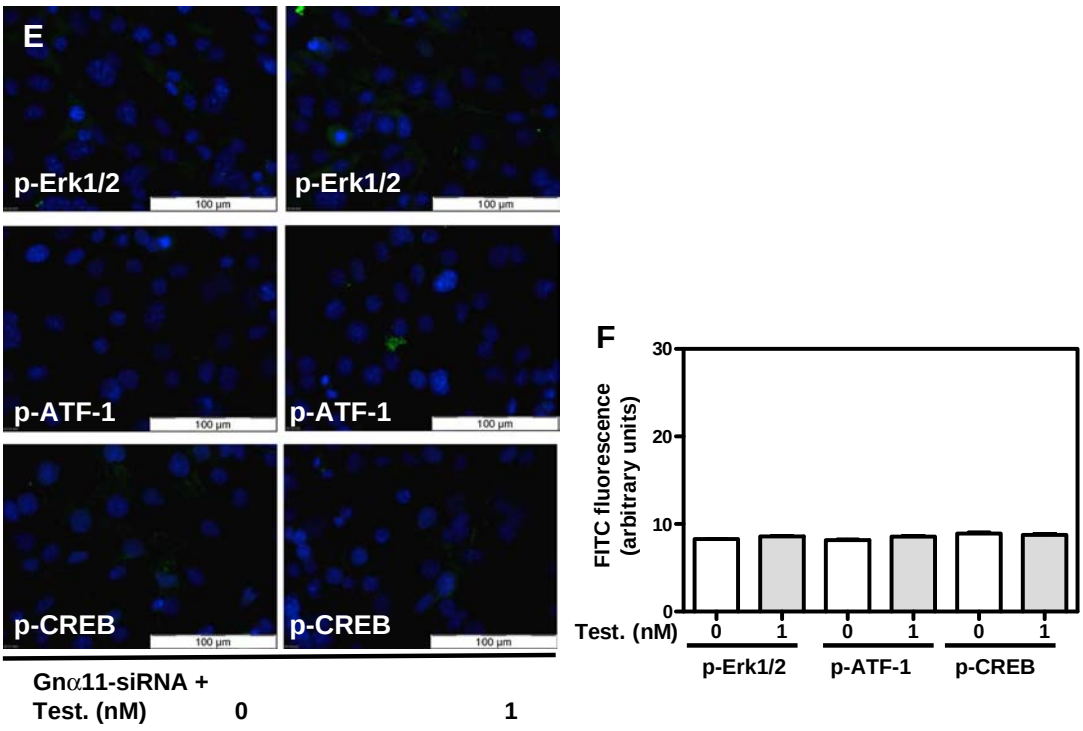

Fig. 7 (*continued*).

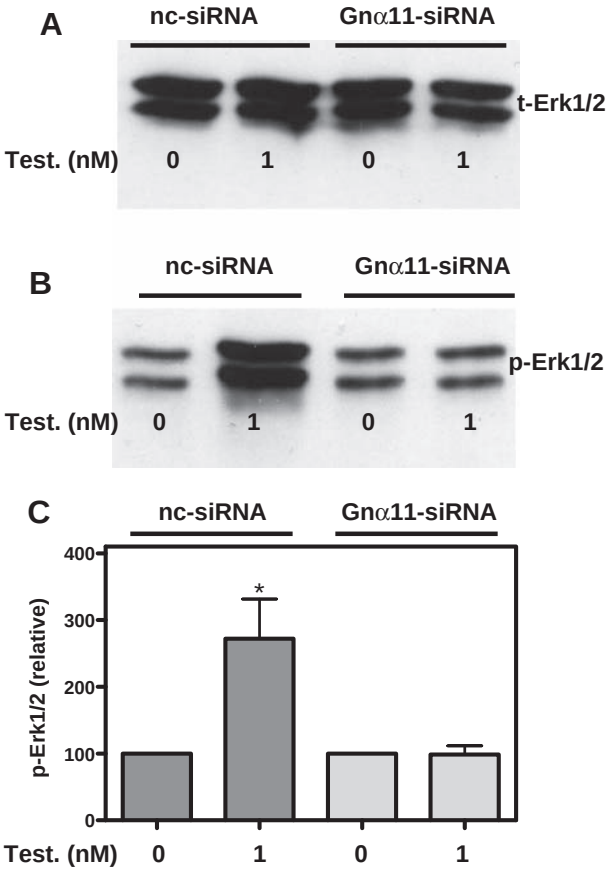

Fig. 8. Western blot analysis of p-Erk1/2 after silencing Gnα11 expression by siRNA. GC-2 cells were treated with nc-siRNA or Gnα11-siRNA followed by 0 or 1 nM testosterone as in Fig. 7. Cell lysates were probed for p-Erk1/2 as in Fig. 4. (A) Total Erk1/2 was not affected by testosterone or Gnα11-siRNA 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

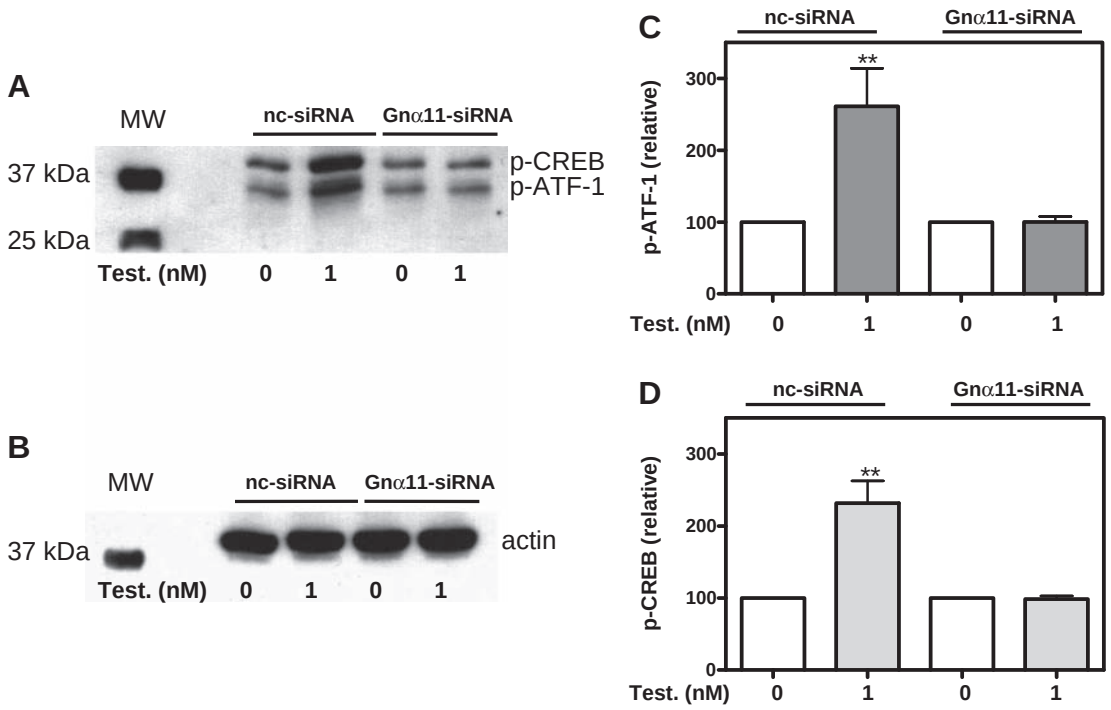

Fig. 9. Western blot analysis of p-ATF-1 and p-CREB after silencing Gnα11 expression by siRNA. Lysates were isolated after treatment of the cells with either nc-siRNA or Gnα11-siRNA. (A) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA but not in cells that had received 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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VI. The third Publication *(Attachment 3)*

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Non-classical testosterone signaling in spermatogenic GC-2 cells is mediated through ZIP9 interacting with Gnα11☆

Mazen Shihan [a], Kai-Hui Chan [b], Lutz Konrad [b], Georgios Scheiner-Bobis [a,b,*]

[a] Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität, Giessen, Germany
[b] Zentrum f. Frauenheilkunde und Geburtshilfe, Fachbereich Medizin, Justus-Liebig-Universität, Giessen, Germany

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ABSTRACT

Although classical and non-classical signaling of testosterone has been documented in several investigations, the nature of the receptor involved in the non-classical pathway remains a source of controversy. While some investigators favor the exclusive participation of the cytosolic/nuclear androgen receptor (AR) in both pathways, others propose a membrane-bound receptor as the mediator of the non-classical testosterone signaling. Evidence is provided here that in the spermatogenic cell line GC-2 the non-classical signaling pathway of testosterone, characterized through the activation of Erk1/2 and transcription factors like CREB or ATF-1, is not mediated through the classical nuclear androgen receptor (AR) but rather by a membrane-associated receptor. This receptor is ZIP9, a Zn^{2+} transporter from the family of the ZRT, IRT-like proteins (ZRT = zinc-regulated transporter; IRT = iron-regulated transporter), which directly interacts with the G-protein Gnα11. siRNA-induced abrogation of the expression of either of these two proteins, whose close contacts are demonstrated by an *in situ* proximity assay, completely prevents all non-classical signaling effects of testosterone addressed. In contrast, silencing of AR expression does not influence the same signaling events. The identification of ZIP9/Gnα11 interactions as the mediators of the non-classical testosterone signaling cascade in spermatogenic GC-2 cells might help to supplement our knowledge concerning the role of testosterone in male fertility and reproduction.

1. Introduction

Steroid hormones interact not only with cytosolic/nuclear steroid hormone receptors (SHRs) to induce a series of well-characterized genomic effects [1–3] but also with membrane-bound SHRs [4–8] to induce rapid events that lead to the activation of cytosolic signaling cascades, such as the Src/PI3K/Akt or the Src/Ras/Raf/Erk1/2 cascade that are normally triggered by growth factors [9,10]. Thus interaction of a membrane-associated subpopulation of the nuclear estrogen receptor ERα with the G-protein Gαi triggers the activation of the Src/PI3K/Akt-cascade leading to activation of endothelial nitric oxide synthase (eNOS) [11,12]. Other membrane-bound SHRs mediating these so-called non-genomic or non-classical effects of steroid hormones are, however, often G-protein-coupled receptors (GPCR) and therefore different from the nuclear SHRs [13–16].

Testosterone acts through this mechanism and induces classical and non-classical signaling [17–19]. Although the cytosolic/nuclear androgen receptor (AR), which serves as a ligand-activated transcription factor [19], is undoubtedly responsible for the classical, genomic actions of testosterone, the nature of the receptor involved in the non-classical pathway is a source of controversy. While some investigators favor the exclusive participation of the well-characterized cytosolic/nuclear AR in both classical and non-classical pathways [20], others propose a membrane-bound receptor, possibly from the family of GPCR, as mediator of several testosterone-induced effects [21–26].

In a recent publication we were able to demonstrate that the non-classical signaling pathway of testosterone in the spermatogenic cell line GC-2 is mediated through the G-protein Gnα11 [27]. In a parallel investigation published subsequently by others it was shown that ZIP9, a Zn^{2+} transporter from the family of the ZRT, IRT-like transporting proteins (ZRT = zinc-regulated transporter; IRT = iron-regulated transporter), binds testosterone with high affinity (Kd = 12.7 nM), activates Erk1/2, and induces testosterone-mediated Zn^{2+} accumulation and apoptotic cell death when expressed in prostate or breast cancer cells [28,29]. The signaling cascade is mediated through the interactions of ZIP9 with stimulatory Gsα proteins, as demonstrated by co-immunoprecipitation, testosterone-induced stimulation of [^{35}S]GTPγS binding to cell membranes from cells expressing ZIP9, decreased specific [^{3}H]testosterone binding to membranes after treatment with excess GTPγS, and through the testosterone-induced elevation of cellular cAMP levels [28,29]. Taking these findings into consideration, we address

☆ Dedicated to the eightieth birthday of my mentor and long-time friend Wilhelm Schoner, whose savvy and scientific curiosity has kept him healthy and young at heart hopefully for many years to come.

* Corresponding author at: Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Str. 100, D-35392 Giessen, Germany.
E-mail address: Georgios.Scheiner-Bobis@vetmed.uni-giessen.de (G. Scheiner-Bobis).

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in the present study whether testosterone-induced non-classical signaling in spermatogenic GC-2 cells is also mediated through ZIP9 and whether these signaling cascades are the result of ZIP9 interactions with Gnα11.

2. Materials and methods

2.1. Cell culture

The murine spermatogenic cell line GC-2 spd (ts) [30] (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 Fig. 1-suppl. regarding choice of concentration). Controls received the equivalent amount of ethanol. After 30 min of incubation (see Fig. 1-suppl. 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 (Merck Chemicals GmbH, Schwalbach, Germany) for 30 min at 0.5 V/cm^2. Desired protein bands were visualized by incubating the membranes with the primary antibody according to the protocol of the providers (Table 1, suppl.), and subsequently the appropriate secondary antibody of an enhanced chemiluminescence solution (made by mixing the buffer with p-coumaric acid, luminol, and H_2O_2[31]). 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. Films were analyzed by the TotalLab gel image analysis software (Biostep, Jahnsdorf, Germany).

2.4. RT-PCR

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). The reverse transcription of the isolated mRNA was carried out by the Reverse Transcription System (Promega) 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, 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′CACAAT GCCAAAGTTGTCA3′ 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 identified by using as forward and reverse primers the oligonucleotides 5′AGCGCAATGCCGCTATGGGG3′ and 5′GTGGGGCTGCCAGCATTGGA3′, respectively. These amplify a 708-bp fragment of mouse AR-specific mRNA localized between bases 1220 and 1927.

Gnα11-specific mRNA/cDNA was amplified using as forward primer the oligonucleotide 5′GAACCGGGAAGAGGTAGGG3′ and as a reverse primer the oligonucleotide 5′GACCAAGTGTGAGTGCAGGA3′. These amplify a 917-bp fragment of mouse Gnα11-specific mRNA localized between bases 70 and 986.

ZIP9-specific mRNA/cDNA was amplified using as forward and reverse primers the oligonucleotides 5′GCAAGGCTGAAAGAAGTGGG3′ and 5′ATTCGATTCCGCTCCAGACC3′, respectively. These amplify a 750-bp fragment of mouse ZIP9-specific mRNA localized between bases 540 and 1289.

2.5. Silencing expression of classical AR, Gnα11 or ZIP9 via siRNA

Expression of AR was silenced by using commercially available siRNA and by following the protocol of the provider (Stealth™ RNAi; Invitrogen, Karlsruhe, Germany). The oligonucleotides used were: oligo pair 1: 5′ACUCGAUCGCAUCAUUGCAUGCAAA3′ and 5′UUUGCA UGCAAUGAUGCGAUCGAGU3′; oligo pair 2: 5′CCCAGAAGAUGACUGU ALJCACACAU3′ and 5′AUGUGUGAUACAGUCAUCUUCUGGG3′; oligo pair 3: 5′CCAGAUUCCUUUGCUGCCUUGUUAU3′ and AUAACAAGGCAG CAAAGGAAUCUGG3′. Control cells were treated with Stealth™ RNAi Negative Control, provided in the kit. Transfection efficiency was estimated by the Block-iT™ Transfection Kit (Invitrogen, Karlsruhe, Germany) according the protocol of the provider.

For silencing Gnα11 expression, cells were treated with commercially available siRNA directed against the expression of Gnα11 (Silencer® Select siRNA; Invitrogen). Control GC-2 cells were treated with the siRNA Negative Control as suggested by the provider (Silencer® Select siRNA; Invitrogen). The oligonucleotide pair used for silencing Gnα11 expression was 5′CAAGAUCCUCUACAAGUAUTT3′ and 5′AUACUUGUAGAGGAUCUUGAG3′ (Gnα11–siRNA).

For silencing ZIP9 expression, GC-2 cells were treated with commercially available siRNA directed against the expression of ZIP9 by following the protocol of the provider (Silencer® Select siRNA; Invitrogen). The oligonucleotide pair used was 5′GGGAAGAUGGAAUUUAGUUTT3′ and 5′AACUAAAUUCCAUCUUCCCTG3′ (ZIP9–siRNA). All other steps were the same as described in the previous paragraph. Control GC-2 cells were treated with the siRNA Negative Control as supplied by the provider (Silencer® Select siRNA; Invitrogen).

After incubation of the GC-2 cells for 72 h with the various siRNA oligo pairs or the negative control siRNA, preparation of samples for

PCR, western blots, immunofluorescence or proximity ligation assay experiments was carried out as described in the previous or subsequent paragraphs.

2.6. Detection of ZIP9/Gnα11-interactions by Duolink™ in situ proximity ligation assay (PLA)

A total of 2×10^4 GC-2 of control cells or cells that had been treated with siRNA against either ZIP9 or Gnα11 were incubated overnight with 0.3 ml medium containing 10% FCS in 8-well chamber slides as described above. The medium was then replaced with 0.1 ml medium containing 1% FCS and incubation was continued for another 24 h. The medium was then aspirated and wells were washed with PBS. After removing the chamber part of the 8-well chamber slide, cells were circled with a hydrophobic barrier pen and then fixed by incubation in 50 μl of 10% neutral buffered formalin for 20 min at room temperature. Cells were then permeabilized by incubation of the slides in 0.25% Triton X-100 for 10 min.

In the following description of the PLA procedure, whenever mentioned, the washing step refers to washing twice for 5 min each in PBS with agitation at room temperature in a Coplin jar, each time followed by the aspiration of the washing solution. After the permeabilized cells were washed, one drop of Duolink II blocking solution was added to each well and incubation continued for 1 h at 37 °C. Thereafter, 20 μl of the antibody against Gnα11 and 20 μl of the antibody against ZIP9, each diluted as recommended by the provider (see Table 1, suppl.), were added to each slide. The wells were then incubated overnight at 4 °C and subsequently washed in PBS as stated above. A total of 40 μl of mixed and diluted *minus* and *plus* PLA probes of the Duolink™ PLA Kit (Sigma-Aldrich, Hamburg, Germany) were then added to each well and incubation proceeded for 1 h at 37 °C. After a further wash, 40 μl of ligation-ligase solution (Duolink™ PLA-Kit) were added to each well and incubation continued for 30 min at 37 °C. Following the washing procedure, 40 μl of amplification-polymerase solution (Duolink™ PLA-Kit) was added to each well and incubation continued for 100 min at 37 °C in the dark. Then the slides were washed and subsequently dipped in PBS for 1 min in a Coplin jar protected from light. A total of 40 μl of mounting medium with DAPI were then added on a cover slip and gently placed over the slide. Images were obtained by an inverse Olympus IX81 microscope equipped with the corresponding fluorescence system (Olympus, Hamburg, Germany).

2.7. Immunofluorescence

After treatment of GC-2 with siRNA to silence either the classical AR, Gnα11 or ZIP9 or (see previous paragraph) the medium was 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 room temperature, the DAPI solution was aspirated and slides were allowed to dry for 15 min before being washed 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 room temperature. The first antibody against either the classical AR, Gnα11, or ZIP9 (Table 1, suppl.), diluted as recommended by the provider, was then added and incubation was continued for 1 day at 4 °C in a humidified chamber. Staining was achieved by incubating for 20 min at room temperature with the secondary antibody [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).

The detection of active (phosphorylated) Erk1/2, CREB or ATF-1 was carried out by following the same fixation-immunostaining protocol described in the previous paragraph, with the difference that the primary antibodies used were specific against either phospho-Erk1/2, phospho-CREB, or phospho-ATF-1 (Table 1, suppl.). The antibody used against phospho-ATF-1 (Epitomics, Burlingame, CA, USA) does not cross-react with p-CREB. For the specific detection of phospho-CREB, an antibody from Cell Signaling Technology (Table 1, suppl.) was used with negligible cross-reaction with p-ATF-1.

Fluorescence within cells was measured by using the software program ImageJ (freely available at http://rsbweb.nih.gov/ij/). Only green fluorescence indicating activated Erk1/2, CREB, or ATF-1 was considered. Cells from 3 independent experiments 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. In Figs. 2–4 all statistical data relate to the untreated controls at 0 nM testosterone. 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 ZIP9, Gnα11, or AR expression by siRNA

After 72 h of incubation of cells with the siRNA oligonucleotides against ZIP9 (ZIP9-siRNA), Gnα11 (Gnα11–siRNA), or AR (AR–siRNA), mRNA was isolated for RT-PCR. RT-PCR revealed that while having no effect on the expression of GAPDH-specific mRNA/cDNA, ZIP9–siRNA reduced the biosynthesis of ZIP9-specific mRNA/cDNA below the detection level (Fig. 1A). At the same time, expression of GAPDH- or ZIP9-specific mRNA was not affected by negative control siRNA (nc-siRNA; Fig. 1A). The RT-PCR result was confirmed by immunofluorescence. Green fluorescence demonstrating the presence of ZIP9 protein in every cell that had been treated with nc-siRNA (Fig. 1B) is absent in cells treated with ZIP9–siRNA (Fig. 1C), demonstrating the successful suppression of ZIP9 expression by the siRNA oligonucleotides used.

Similarly, treatment of GC-2 cells with Gnα11-specific siRNA reduced the expression of its mRNA/cDNA (Fig. 1D) and protein (Fig. 1F), but they were unaffected by nc-siRNA (Fig. 1D and E), indicating the specificity of the silencing reaction. This is also underlined by the fact that GAPDH-specific mRNA/cDNA was comparable in cells that had been treated with either nc-siRNA or Gnα11–siRNA (Fig. 1D).

The suppression of AR-specific mRNA/cDNA expression was most successful when oligo pair 3 (op3) was applied (Fig. 1G). Although a faint amplification product representing AR-specific mRNA/cDNA was still seen in the presence of op3, immunofluorescence experiments showed complete suppression of the AR protein expression: whereas green fluorescence indicating the expression of the AR protein was visible in every GC-2 cell that had been treated with nc-siRNA (Fig. 1H), it was entirely absent after treatment of the cells with AR-siRNA (Fig. 1I). It is therefore likely that the weak AR-specific signal seen after treatment with AR-siRNA op3 (Fig. 1G) is the result of the strong amplification effects of the RT-PCR.

3.2. Non-classical testosterone signaling in GC-2 cells in the presence or absence of ZIP9, Gnα11, or AR

In the non-classical action of testosterone, activation of the Src/Ras/Raf/Erk1/2 signaling cascade leads to the activation of the transcription factor CREB. We therefore addressed a possible testosterone-induced activation of Erk1/2, CREB, and ATF-1 in GC-2 cells. Like CREB, ATF-1 is a member of the bZIP superfamily of transcription factors that stimulate transcription when activated by phosphorylation at either Ser63 (ATF-1)

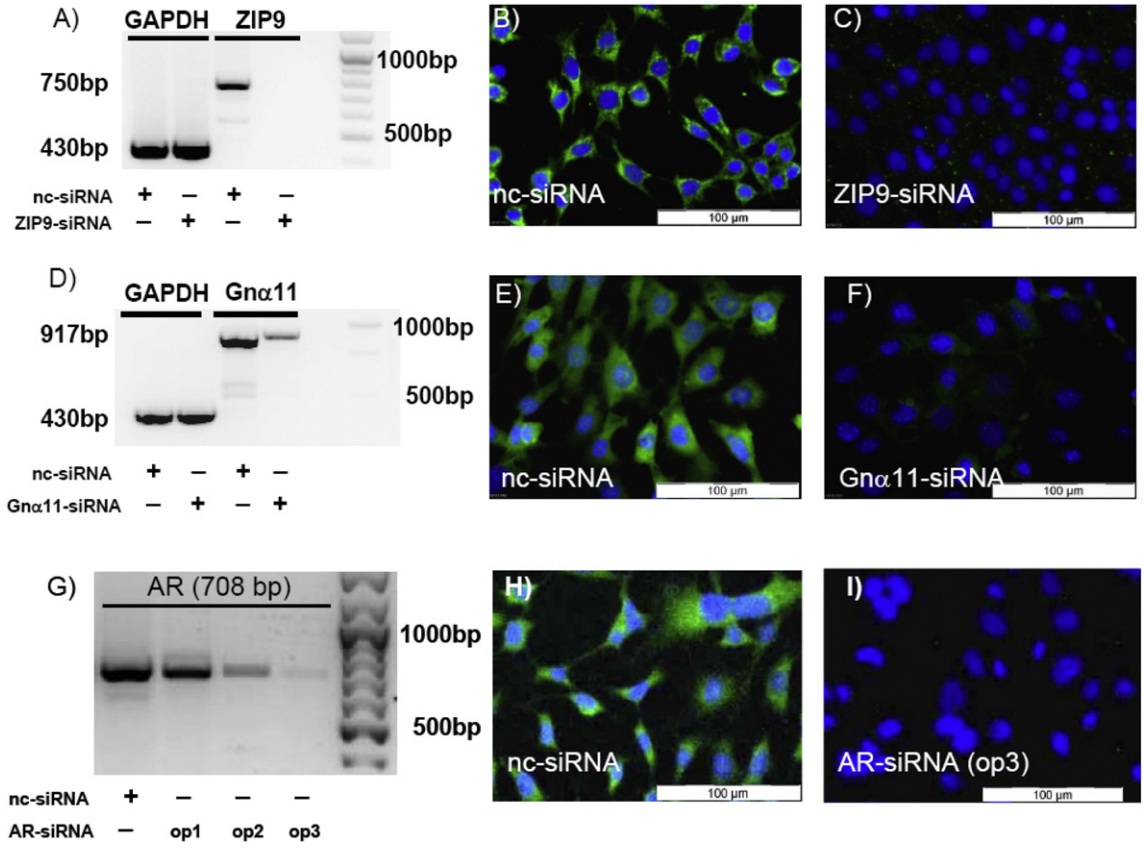

Fig. 1. Silencing expression of ZIP9, Gna11 or classical AR by siRNA. In the following photomicrographs, blue coloring refers to DAPI-stained nuclei and green indicates the secondary Alexa Fluor 488-labeled goat anti-rabbit IgG bound to ZIP9-, Gnα11- or AR-specific primary antibodies. Treatment of cells with negative-control siRNA (nc-siRNA) did not affect the expression of either ZIP9- (A) Gnα11- (D) or AR-specific (G) mRNA/cDNA or the expression of ZIP9 (B) Gnα11 (E) or AR (H) proteins. Application of specific siRNAs against ZIP9 (ZIP9–siRNA), Gnα11 (Gnα11–siRNA), or AR (AR-siRNA; op3) considerably reduced the RT-PCR signals for ZIP9- (A), Gnα11- (D), or AR-specific (G) mRNA/cDNA fragments (750, 917, or 708 bp, respectively) and abrogated the expression of the corresponding proteins (C: ZIP9; F: Gnα11; I; AR).

or Ser133 (CREB). Simultaneous activation of the two related transcription factors has been shown previously [32], and we investigated whether testosterone might act on GC-2 cells in a similar way.

Control cells and cells that had been treated with nc-siRNA, ZIP9–siRNA, Gnα11–siRNA, or AR-siRNA were incubated with either 0 or 1 nM testosterone for 30 min and then subjected to fixation and immunostaining. Phosphorylated forms of Erk1/2 (Fig. 2), CREB (Fig. 3), or ATF-1 (Fig. 4) were detected by using appropriate antibodies (Table 1, suppl.). In control cells and in cells treated with nc-siRNA 1 nM testosterone causes a significant stimulation (= phosphorylation) of Erk1/2 (Fig. 2), CREB (Fig. 3), or ATF-1 (Fig. 4). Similar results were obtained with cells that were treated with AR-siRNA to silence AR expression. Testosterone induced a clear and significant activation of Erk1/2 (Fig. 2), CREB (Fig. 3) or ATF-1 (Fig. 4) that was not affected by the abrogation of AR expression.

In contrast, suppression of either ZIP9 or Gnα11 expression by the corresponding siRNAs resulted in complete obliteration of the testosterone-induced activation of Erk1/2 (Fig. 2), CREB (Fig. 3), or ATF-1 (Fig. 4), indicating the importance of both ZIP9 and Gnα11 for the non-classical testosterone signaling pathway.

3.3. Detection of p-Erk1/2, p-ATF-1, and p-CREB in western blots in the presence or absence of ZIP9, Gnα11, or AR

Since immunofluorescence photomicrographs can only show cells or proteins that are within the optical field of the microscope, western blots were carried out to obtain a representative average by measuring the testosterone action on all cells. Testosterone effects on GC-2 cells treated with nc-siRNA were compared with its effects on cells treated with ZIP9–siRNA, Gnα11–siRNA, or AR-siRNA. Treatment of GC-2 cells with ZIP9–siRNA completely impaired the ability of testosterone to induce activation of Erk1/2 (Fig. 5A–C), which is consistent with the results shown in Fig. 2. The total amount of Erk1/2 was not affected by the steroid hormone or the ZIP9–siRNA (Fig. 5A).

Similarly, in the absence of ZIP9 testosterone failed to cause activation of either CREB or ATF-1. In western blots with an antibody that cross-reacts with p-CREB and p-ATF-1, we observed significant activation of both transcription factors following 30 min of incubation with 1 nM testosterone only when cells were treated with nc-siRNA (Fig. 5E–G). Treatment of cells with ZIP9–siRNA abrogated CREB and ATF-1 stimulation (Figs. 5E–G), consistent with the results shown in Figs. 3 and 4. Total actin was not affected by the nc-siRNA or ZIP9–siRNA (Fig. 5D), indicating that the loss of p-CREB or p-ATF-1 in ZIP9–siRNA treated cells was not due to an overall reduction in protein expression.

Inhibition of Gnα11 expression had effects on signaling that were similar to those produced by inhibition of ZIP9 expression. Whereas treatment of cells with nc-siRNA did not impair the significant testosterone-induced stimulation of Erk1/2 (Fig. 6B and C), exposure of the GC-2 cells to Gnα11–siRNA completely blocked the stimulation of the kinase (Fig. 6B and C). Total Erk1/2 was not affected by nc-siRNA or Gnα11–siRNA (Fig. 6A), indicating that the loss of Erk1/2 activation in Fig. 6B is not the result of reduced Erk1/2 expression. All of these results are consistent with the observations presented in Fig. 2.

Activation of CREB or ATF-1 was also strongly affected when Gnα11 expression was suppressed by Gnα11–siRNA. Consistent with the results

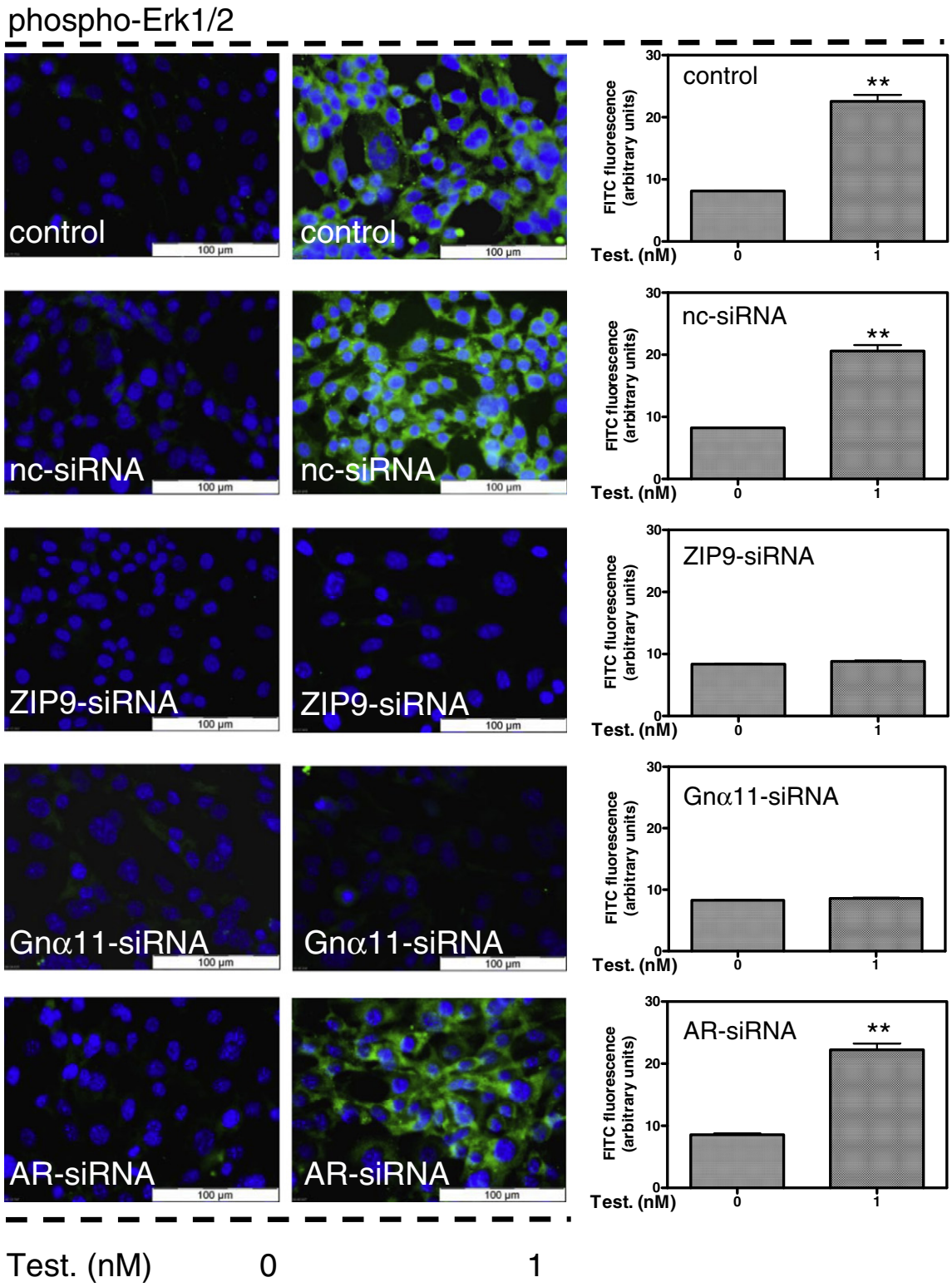

Fig. 2. Activation of Erk1/2 by testosterone. All cells shown were incubated with a primary antibody against the phospho-Erk1/2 and a fluorescent secondary antibody (Alexa Fluor 488). Thus, phospho-Erk1/2 appears green and DAPI-stained nuclei appear blue. The left column shows phospho-Erk1/2 in the absence of testosterone, the middle column the same protein after stimulation with 1 nM testosterone, and the left column the statistical analysis of the green fluorescence ($n = 3$; 19–28 cells counted from each experiment; means ± SEM; ** = $p \leq 0.01$). Activation (phosphorylation) of Erk1/2 was only seen after testosterone stimulation of control cells (control), cells treated with negative control siRNA (nc-siRNA), and AR specific siRNA (AR-siRNA). Treatment of cells with siRNA against either ZIP9 (ZIP9–siRNA) or Gnα11 (Gnα11–siRNA) entirely abrogated the testosterone-induced stimulation of Erk1/2.

summarized in Figs. 3 and 4, testosterone failed to activate either of the two transcription factors in Gnα11–siRNA-treated cells (Fig. 6E, F and G). nc-siRNA did not prevent the testosterone-induced stimulation of CREB or ATF-1 (Fig. 6E–G), underlining the importance of this G-protein for the non-classical signaling pathway of testosterone. Total actin was not affected by either of the two siRNAs (Fig. 6D).

In contrast with the results obtained after silencing ZIP9 or Gnα11 expression, silencing AR expression had no significant effect

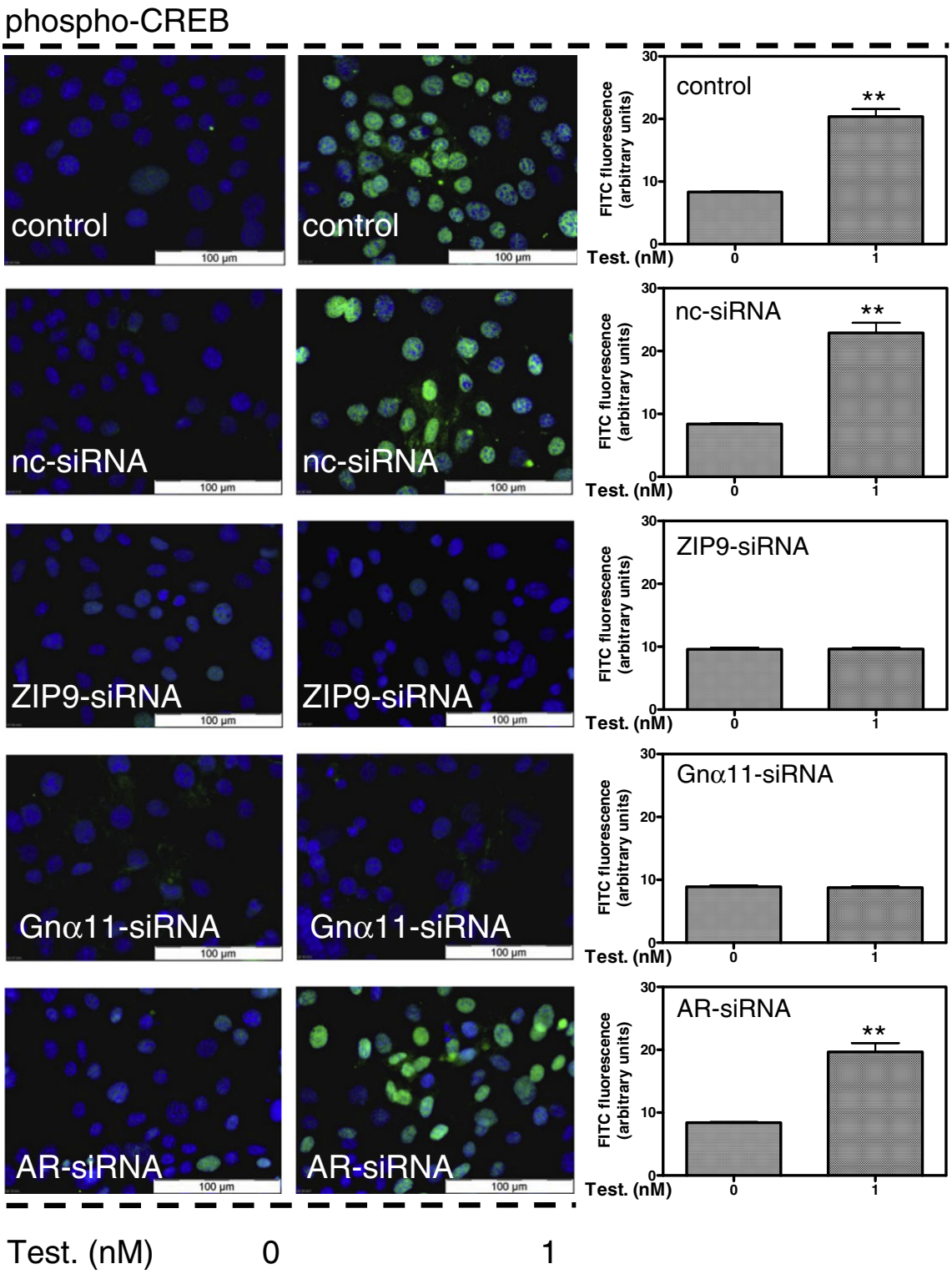

Fig. 3. Activation of CREB by testosterone. The arrangement of the figure is the same as in Fig. 2. Activated (phosphorylated) CREB is indicated by green fluorescence and nuclei are stained blue. Testosterone-induced activation of CREB (phospho-CREB) was observed in control cells (control), in cells treated with nc-siRNA and in cells treated with AR-specific siRNA (AR-siRNA). Treatment of cells with siRNA against either ZIP9 (ZIP9–siRNA) or Gnα11 (Gnα11–siRNA) completely inhibited the testosterone-induced stimulation of CREB. For statistical analysis of the green fluorescence data from three independent experiments were considered (n = 3; 22–33 cells counted from each experiment; means ± SEM; ** = p ≤ 0.01).

on testosterone signaling. Treatment of GC-2 cells with AR-siRNA did not impair the ability of testosterone to induce significant activation of Erk1/2 (Fig. 7B, C). The total amount of Erk1/2 was not affected by the steroid hormone (Fig. 7A). Similarly, in the absence of AR testosterone activation of ATF-1 and CREB was unchanged (Fig. 7E–G).

3.4. Demonstration of direct interactions between ZIP9 and Gnα11

All the results obtained thus far indicate that the non-classical signaling pathway of testosterone is not triggered by the interaction of the steroid with the known cytosolic/nuclear AR but rather through its

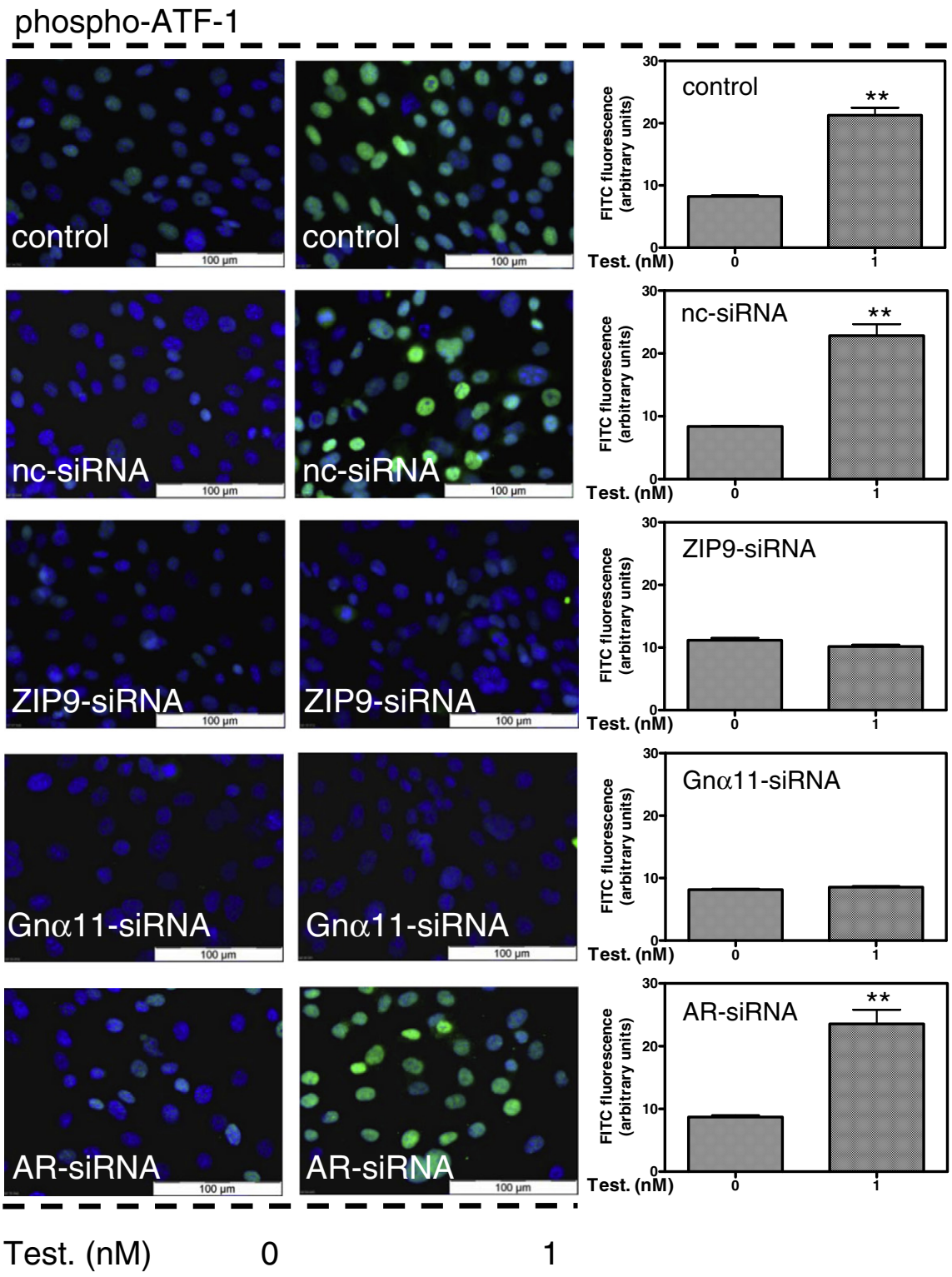

Fig. 4. Activation of ATF-1 by testosterone. The arrangement of the figure is the same as in Fig. 2. Activated (phosphorylated) ATF-1 appears green and nuclei are stained blue. ATF-1 stimulation (phospho-ATF-1) by testosterone was observed in control cells (control), in cells treated with nc-siRNA and in cells treated with AR-siRNA. Treatment of cells with siRNA against either ZIP9 (ZIP9–siRNA) or Gnα11 (Gnα11–siRNA) completely inhibited the testosterone-induced stimulation of CREB. For statistical analysis of the green fluorescence data from three independent experiments were considered (n = 3; 17–35 cells counted from each experiment; means ± SEM; ** = $p \leq 0.01$).

interactions with ZIP9 and Gnα11. Do these two proteins, however, interact with each other or are they involved in different testosterone-triggered pathways? This question was addressed by a rather new method termed *in situ* proximity ligation assay (PLA). In order to address possible interactions between ZIP9 and Gnα11, the two proteins were targeted with a rabbit IgG and a mouse IgG, respectively. When both primary antibodies were present, red fluorescent dots indicating neighboring ZIP9 and Gnα11 were seen in each of the cells, suggesting a direct interaction of the two proteins (Fig. 8A). The fact that not a single red dot was observed in any of the cells when the ZIP9 or Gnα11

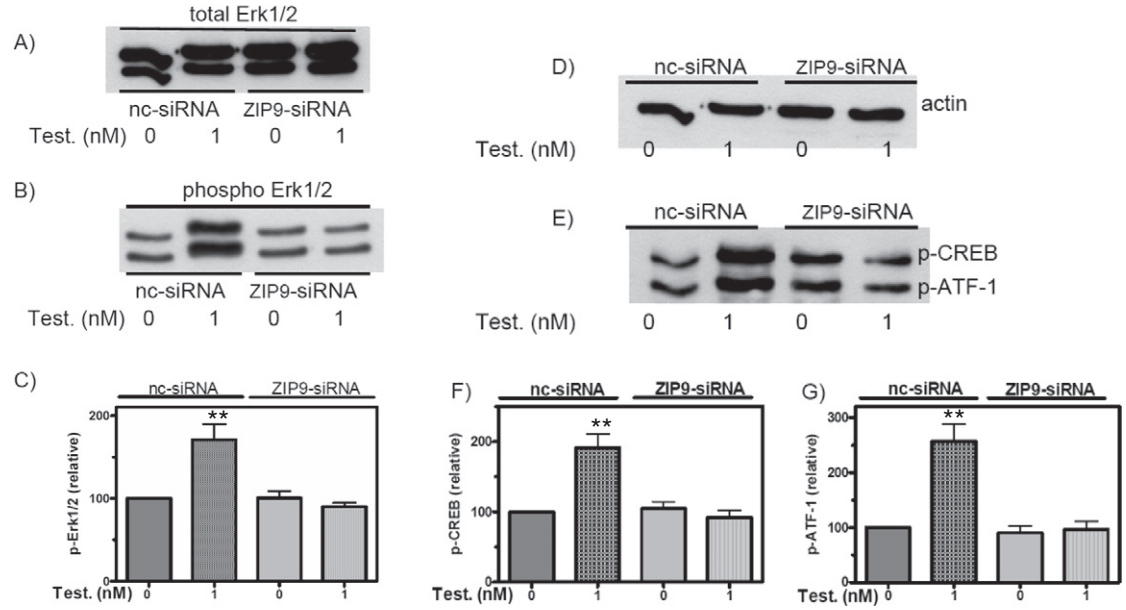

Fig. 5. Western blot analysis of p-Erk1/2, p-CREB, and p-ATF-1 after silencing ZIP9 expression by siRNA. (A) The amount of total Erk1/2 was not affected during the course of the incubation and served as a gel-loading control. (B) Testosterone stimulated Erk1/2 activation in GC-2 cells treated with nc-siRNA but had no effect in cells that had been treated with ZIP9–siRNA. (C) For statistical analysis, data were corrected for the amount of total Erk1/2 as shown in panel (A) ($n = 3$; means ± SEM; ** = $p \leq 0.01$). (D) Expression of total actin was not affected by the treatment of the cells with either type of siRNA or testosterone. (E) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA but not in cells that had received ZIP9–siRNA. (F and G) For statistical analysis data were corrected for the amount of total actin as shown in B ($n = 3$; means ± SEM; ** = $p \leq 0.01$).

expression was abrogated by means of siRNA (Fig. 8, B and C) underlines the specificity of the PLA assay and supports the idea of direct interaction of ZIP9 and Gnα11 proteins.

4. Discussion

The non-classical signaling pathway of testosterone in Sertoli cells that leads to the activation of the Ras/Raf/Erk1/2/CREB cascade [20,33] is thought to be mediated through the classical cytosolic/nuclear AR, since abrogation of its expression by siRNA impairs the testosterone-induced signaling cascade [34]. In the spermatogenic cell line GC-2, however, this signaling cascade is not affected by silencing AR expression but rather by silencing the expression of the G-protein Gnα11 [27], pointing towards the involvement of a membrane-bound GPCR as the origin of the non-classical testosterone-induced cell signaling. Although this discrepancy might be due to the different cell types used

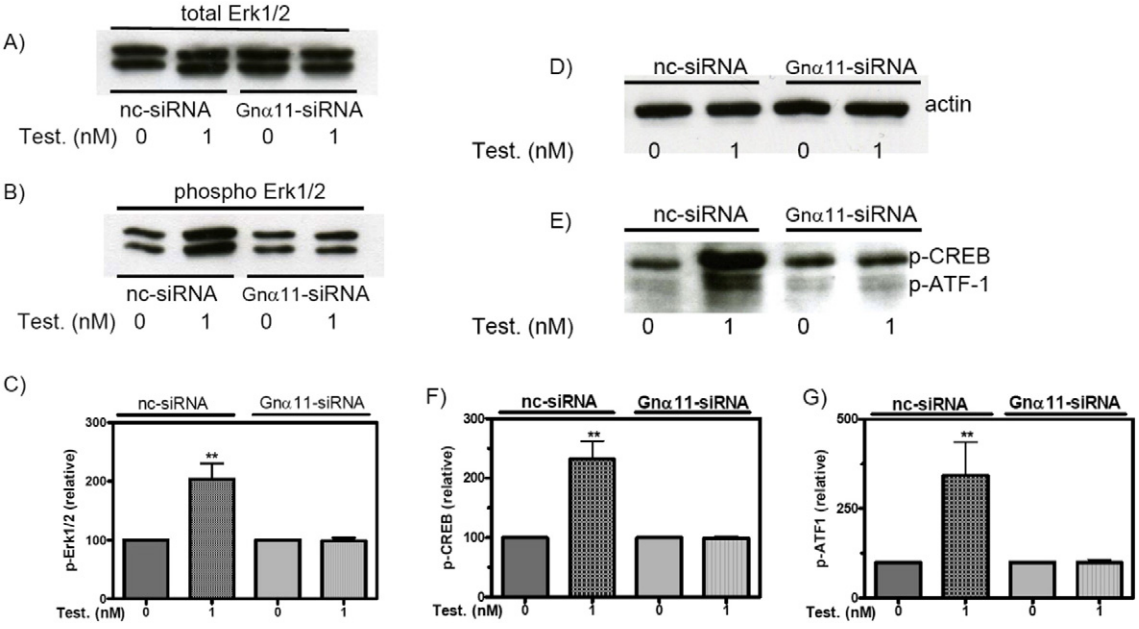

Fig. 6. Western blot analysis of p-Erk1/2, p-CREB and p-ATF-1 after silencing Gnα11 expression by siRNA. (A) The amount of total Erk1/2 was not affected by incubation with siRNA or by testosterone. (B) Testosterone stimulated Erk1/2 activation in GC-2 cells treated with nc-siRNA but had no effect in cells treated with Gnα1-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; * = $p \leq 0.05$). (D) Expression of total actin was not affected by the treatment of the cells with either type of siRNA or testosterone. (E) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA but not in cells exposed to Gnα11-siRNA. (F and G) For statistical analysis data were corrected for the amount of total actin as shown in (D) ($n = 3$; means ± SEM; ** = $p \leq 0.01$).

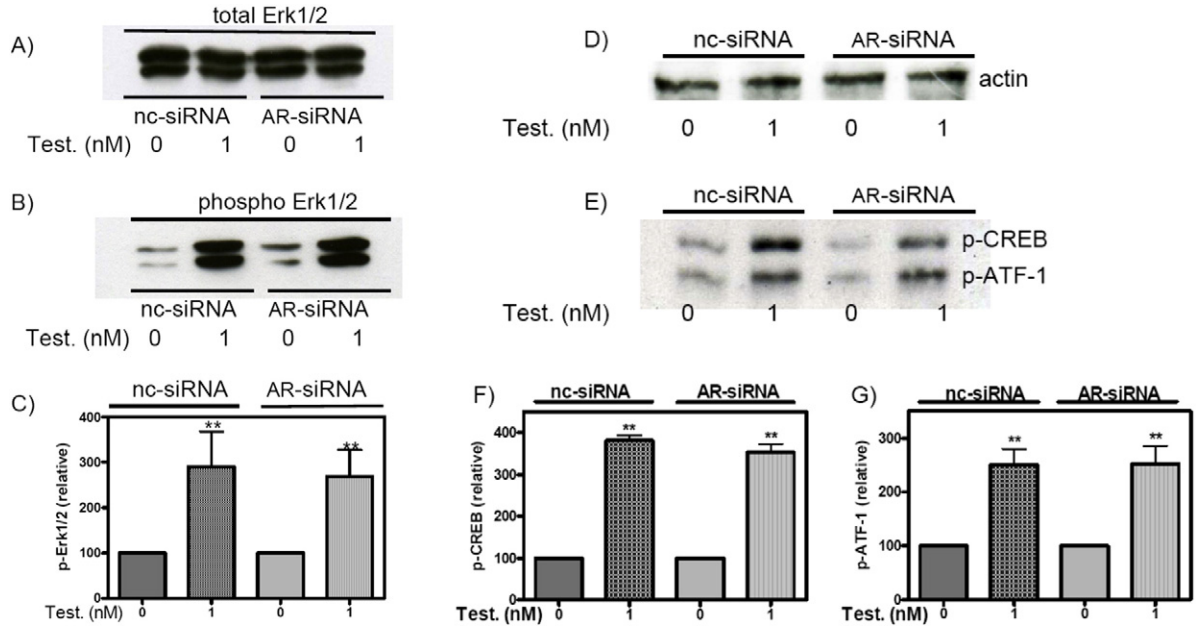

Fig. 7. Western blot analysis of p-Erk1/2, p-CREB and p-ATF-1 after silencing AR expression by siRNA. Cells were treated with either nc-siRNA or with AR-siRNA as described under "Materials and methods". After 30 min of incubation in the presence or absence of 1 nM testosterone, cell lysates were prepared 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; * = $p \leq 0.05$). (D) Incubation with 1 nM testosterone had no effect on total actin. (E) Testosterone stimulated p-CREB and p-ATF-1 formation in cells treated with nc-siRNA or AR-siRNA to the same extent. (G) Analysis of pooled data like those shown in B ($n = 3$; means ± SEM; ** = $p \leq 0.01$). The data in the bar graph were corrected for the amount of total actin as shown in (A), which was used as a gel loading control.

in the two investigations, the identification of a GPCR or some other membrane-bound receptor interacting with Gnα11 as the mediator of the non-classical testosterone signaling pathway would help to supplement current knowledge about the action of this steroid in spermatogenic cells of the male reproductive system.

An opportunity to address this question arose after the identification of ZIP9 as a membrane-bound receptor for testosterone that, when expressed in granulosa/theca cells and also in prostate and breast cancer cells, mediates testosterone-induced non-classical signaling and increases intracellular Zn^{2+} that induces apoptosis [28,29]. Zinc has multiple effects on prostate physiology. Whereas healthy prostate epithelial cells are characterized by the accumulation of high levels of zinc, a decline in cellular zinc concentration is associated with malignant transformation and constitutes a hallmark of prostate cancer [35]. This metabolic transformation is associated with down-regulation of zinc transporters like hZIP1 [36]. Once neoplastic, prostate cancer cells become sensitive to Zn^{2+}-induced apoptosis mediated through increased expression of pro-apoptotic Bax [37]. ZIP9, by being a Zn^{2+} transporter, could very well be involved in testosterone-induced non-classical signaling that increases intracellular Zn^{2+} and induces apoptosis in neoplastic cells or in granulosa/theca cells. Is ZIP9, however, the same receptor that is involved in the non-classical signaling cascade of testosterone in spermatogenic GC-2 cells? And, if yes, does it interact with Gnα11?

We addressed these questions by investigating testosterone-induced signaling in GC-2 cells after silencing the expression of ZIP9, Gnα11, or AR by siRNA. Testosterone-induced Erk1/2, CREB, or ATF-1 activation is highly significant in all control cells and in AR-siRNA-treated cells. In cells treated with siRNA to silence the expression of either ZIP9 or Gnα11, however, testosterone fails to induce activation of any of the above components, indicating the requirement for both of these proteins in the non-classical signaling pathway.

Whereas the results clearly show that ZIP9 and Gnα11 are both mediators of testosterone-induced signaling, the question remained whether the two proteins directly interact with each other to mediate the signals. As clearly shown this seems to be the case. Application of the proximity ligation assay, a method often used to identify cooperating and interacting proteins [38,39], demonstrated the close contact between ZIP9 and stimulatory Gnα11 (Fig. 8), consistent with the idea that these two proteins interact to mediate testosterone-induced non-classical signaling and supporting earlier investigations demonstrating interactions of ZIP9 with the stimulatory Gαs by co-immunoprecipitation [28].

The confirmation of ZIP9 as the mediator of the non-classical signaling pathway of testosterone in spermatogenic GC-2 cells should help us to better understand the manifold effects of this steroid hormone. Non-classical action of testosterone on cells of the male reproductive system is essential for spermatogenesis and the differentiation of spermatogonia to spermatozoa [20]. Activation of cyclic AMP response element binding protein (CREB) in testicular Sertoli cells, which is required for the survival of spermatocytes and the production of mature spermatozoa [40], is triggered by testosterone interactions with the AR *via* the activation of the c-Src/c-Raf/Erk1/2 signaling cascade of the non-classical testosterone signaling pathway [20,26,41]. Spermatogenesis also depends on the activation of Erk1/2 and other mitogen-activated protein kinases [42,43]. In particular, Erk1/2 activation is an absolute requirement for the production of haploid spermatozoa [44,45]. The finding that ZIP9 is involved in these signaling events helps to supplement our knowledge concerning the actions of testosterone and provides a clearer picture of the involvement of this steroid hormone in the regulation of male fertility and reproduction.

Confirmation of ZIP9/Gnα11 interactions as an alternative route of androgen-induced signaling in further cell types and tissues might help not only to supplement our knowledge concerning actions of testosterone but also to reveal new signaling pathways triggered by the steroid.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2015.07.013.

Disclosure statement

The authors declare no conflict of interest associated with this study.

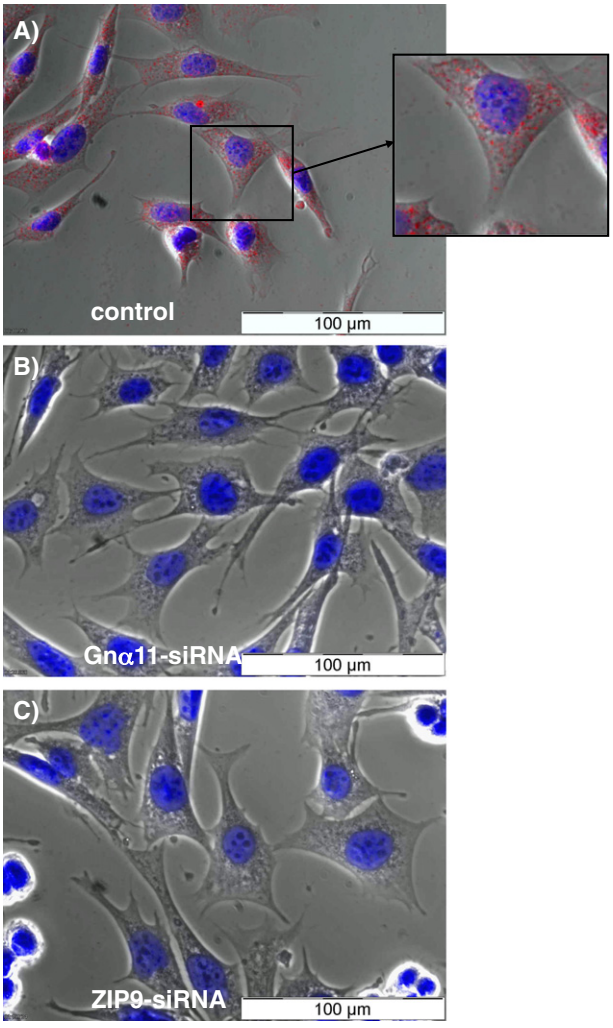

Fig. 8. Demonstration of ZIP9/Gnα11 interactions by the proximity ligation assay. Cells plated on 8-well chambers slides were fixed as stated under "Materials and methods" to preserve the reaction status and transient interactions. The primary antibodies of the ZIP9 and Gnα11 were added followed by Duolink secondary PLA probes. The results were then visualized using a fluorescence microscope. (A) The red points in control cells indicate that the distance between ZIP9 and Gnα11 is less than 40 nm. (B) When Gnα11 or (C) ZIP9 expression was suppressed by siRNA, no red dots were identified in any of the cells, thus underlining the specificity of the reaction in (A) demonstrating the close contacts between ZIP9 and Gnα11.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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