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Photo-DHEA—A functional photoreactive dehydroepiandrosterone (DHEA) analog

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ABSTRACT

The steroid hormone dehydroepiandrosterone (DHEA) has beneficial effects on vascular function, survival of neurons, and fatty acid metabolism. However, a specific receptor for DHEA has not been identified to date. Here, we describe the synthesis of a photoreactive DHEA derivative (Photo-DHEA). In Photo-DHEA, typical characteristics of DHEA are conserved: (i) a "planar" tetracyclic ring system with a Δ^5 double bond, (ii) a 3β-hydroxyl group, and (iii) a keto group at C17. In cell-based assays, Photo-DHEA showed the same properties as DHEA. We conclude that Photo-DHEA is suitable for radioiodination to yield a tool for the identification of the elusive DHEA receptor.

1. Introduction

The steroid dehydroepiandrosterone (Fig. 1(1)) is produced by the adrenals and the central nervous system [1]. Besides its role as a precursor of androgens and estrogens, DHEA has been reported to be neuroprotective [2] and to have beneficial effects on diabetes, atherosclerosis, and obesity [3–5]. Declining DHEA and DHEA sulfate (DHEAS) levels during aging is associated with pathophysiological effects such as neuronal degeneration [6]. Although several DHEA activated intracellular pathways have been characterized, a specific DHEA binding receptor has not been identified to date. Photoreactive steroid analogs are appropriate tools for analysis or identification of steroid binding proteins. The formation of covalent bonds between the photoreactive steroid and the protein upon irradiation allows the application of denaturating preparative and analytical methods such as SDS polyacrylamide gelelectrophoresis and mass spectrometry.

Here, we report the synthesis of the photoreactive DHEA-analog Photo-DHEA (Fig. 1(2)). As starting compound we chose 5-androsten-3β-ol-7,17-dione-7-O-carboxymethyloxime (DHEA-CMO (8), Scheme 1) in order to retain an unaltered DHEA core structure with a hydroxyl group at C3 in β-configuration, a Δ^5 double bound and a keto group at C17. DHEA-CMO was used successfully for the synthesis of the fluorescent DHEA analog DHEA-Bodipy (Fig. 1(3)), which we introduced previously as a tool to study intracellular trafficking and localization of DHEA [7]. As photoreactive group, we employed the aryl azide 4-azidosalicylic acid that forms a nitrene upon irradiation which can react with appropriate nucleophiles such as primary amines, and cysteinyl or histidyl residues of proteins. In Photo-DHEA, DHEA-CMO is connected with 4-azidosalicoyl-ethylenediamine by an amide bond. The long spacer between DHEA and the aryl azide provides a flexible link between the steroid hormone and the photoreactive group. Thereby, potentially interfering effects of the photoreactive aryl azide on the functions of DHEA should be minimized. Moreover, a certain flexibility between the photoreactive group and the steroid is beneficial because this might enable the labeling of several amino acids at the ligand binding site [8]. Aryl azides are the most commonly used photoactivatable reagents because they are easy to synthesize, relatively stable, have high extinction coefficients, and a relatively short half-life upon irradiation [9].

The biological functionality of Photo-DHEA was confirmed by two cell-based assays: displacement of DHEA-Bodipy from specific DHEA binding-sites on neuronal cells and induction of cAMP synthesis by Photo-DHEA in liver cells. Our results indicate that radioiodinated Photo-DHEA should be a useful tool for photoaffinity labeling and identification of DHEA receptor(s).

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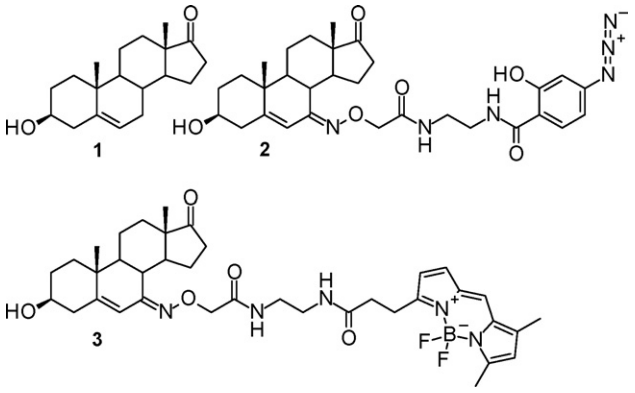
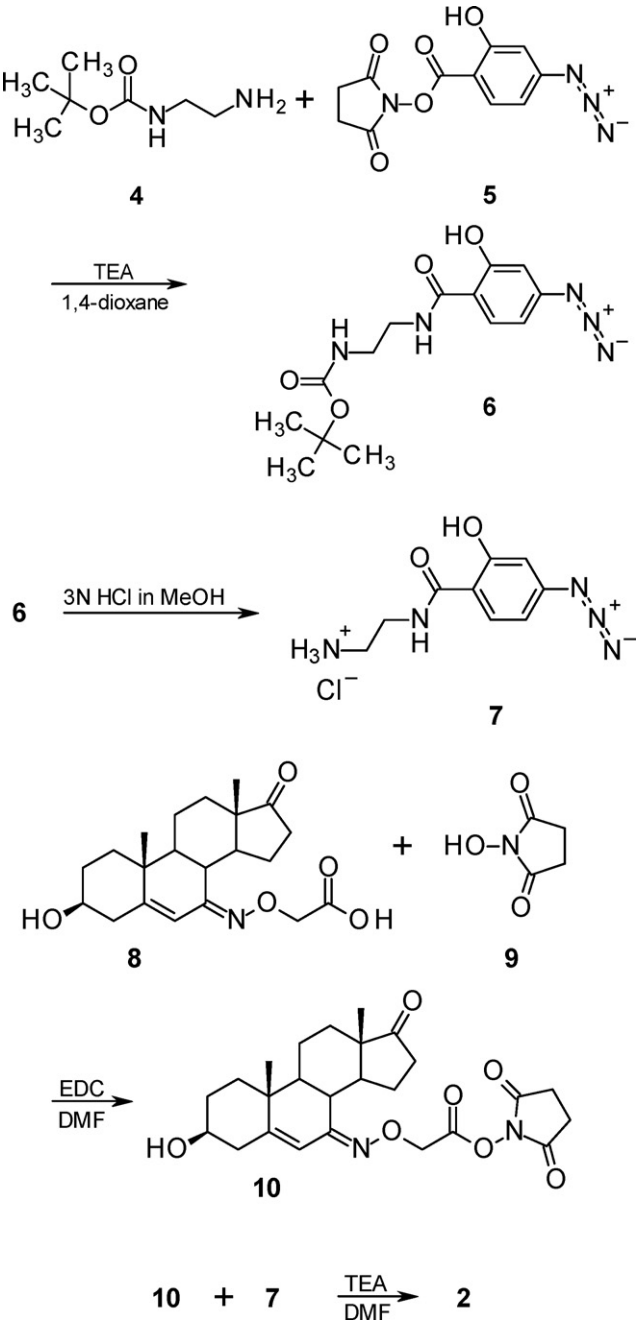


Fig. 1. DHEA (1), Photo-DHEA (2), and DHEA-Bodipy (3).



Scheme 1. Synthesis of Photo-DHEA.

2. Experimental

2.1. General methods

NMR spectra were recorded on Bruker AC-300 instrument in $CDCl_3$ at 300 MHz unless otherwise indicated. Chemical shift values are given in δ (ppm) and coupling constants are given in Hz. ESI mass spectra were recorded on Micromass Q-TOF Ultima API instrument. IR spectra were recorded on Bruker Tensor 27 instrument. Analytical thin layer chromatography (TLC) was performed on 0.5 mm thick silica gel 60 F_{254} plates. Compounds were revealed by UV light (λ = 254 nm) or by staining with ninhydrin. Commercial reagents were used without further purification unless otherwise noted.

2.2. Chemical synthesis

All synthesis steps were performed under dim light. The synthetic pathways are depicted in Scheme 1.

2.2.1. 4-Azidosalicoyl-N-Boc-ethylenediamine (**6**)

Reaction and purification of **6** were performed according to Bidasee et al. [10]. 500 mg (1.81 mmol) of **5** was dissolved in dioxane (4 ml). 320 mg (2.0 mmol) of N-Boc-ethylenediamine (4) and 2.5 ml triethylamine were added. The reaction mixture was stirred under N_2 at room temperature for 3.5 h. The mixture was evaporated, the solid was dissolved in 10 ml of chloroform and chromatographed on a silica gel column (33 cm, Ø 2.5 cm) with chloroform/methanol (99/1) to afford 408.05 mg (1.3 mmol, 70%) of **6** as colorless crystals.

^{1}H NMR ($CDCl_3$, 300 MHz), δ [ppm]: 1.41 (s, 9H, tert-Butyl), 3.41–3.42. (t, 2H, CH_2–N), 3.46–3.48. (t, 2H, CH_2–N), 5.06 (t, 1H, –OH), 6.46 (dd, 1H, ar), 6.58 (d, 1H, ar), 7.44 (d, 1H, ar), 7.84 (s, –NH–) IR (nujol) $\tilde{\nu}$ [cm^{-1}] = 2120 (azide) (compare: IR (KBr) $\tilde{\nu}$ [cm^{-1}] = 2130 (N_3, **7**) [11], IR (nujol) $\tilde{\nu}$ [cm^{-1}] 2102 (azide, N-(4-azido-salicyloyl-β)-alanine ethyl ester)) [10].

TLC: detection UV and ninhydrin (yellow spot). R_f: 0.2 ($CHCl_3$/MeOH (99:1, v/v)), 1.0 (ethylacetate).

2.2.2. 4-Azidosalicoyl-ethylenediamine hydrochloride (**7**)

400 mg (1.24 mmol) of **6** were dissolved in 8 ml of HCl/methanol (1/4), stirred under N_2 at room temperature for 20 h, and allowed to stand at 4 °C [12]. The resulting crystals were filtered, washed with diethylether and dried to afford 237.1 mg (0.9 mmol, 74%) of **7** as colorless crystals.

^{1}H NMR (CD_3OD, 300 MHz), δ [ppm]: 2.97–3.01 (t, 2H, CH_2–N), 3.51–3.57 (q, 2H, CH_2–NH), 6.63 (d, 1H, ar), 6.65–6.69 (dd, 1H, ar), 7.97 (d, 1H, ar), 9.04–9.07 (m, –NH–). *Note*: lack of the tert-butyl singlet (1.41 ppm) indicates successful N-Boc deprotection.

TLC: detection UV and ninhydrin (red-purple spot). R_f: 0.3 (ethylacetate).

2.2.3. Photo-DHEA (**2**) (4-azido-2-hydroxy-N-(2-(2-((E)-((3S,8R,9S,10R,13S,14S)-3-hydroxy-10,13-dimethyl-17-oxo-3,4,9,11-tetrahydro-1H-cyclopenta[a]phenanthren-7(2H,8H,10H,12H,13H,14H,15H,16H,17H)-ylidene)aminooxy)acetamido)ethyl)benzamide)

To a solution of 200 mg (0.53 mmol) of DHEA-CMO (**8**) in 1.5 ml of DMF, 124 mg (1.08 mmol) of **9** and 104 mg (0.54 mmol) of EDC were added similar to [13]. The reaction mixture was stirred under N_2 at room temperature for 22 h. 136 mg (0.53 mmol) of **7** in 0.5 ml of DMF and 80 μl of triethylamine were added. The reaction mixture was stirred at room temperature for 24 h, filtrated and diluted with H_2O. The mixture was extracted with ethylacetate. The combined organic layers were washed with saturated NaCl solution, dried with Na_2SO_4, filtered and evaporated. The resulting yellow oil was chromatographed on a silica gel column (33 cm,

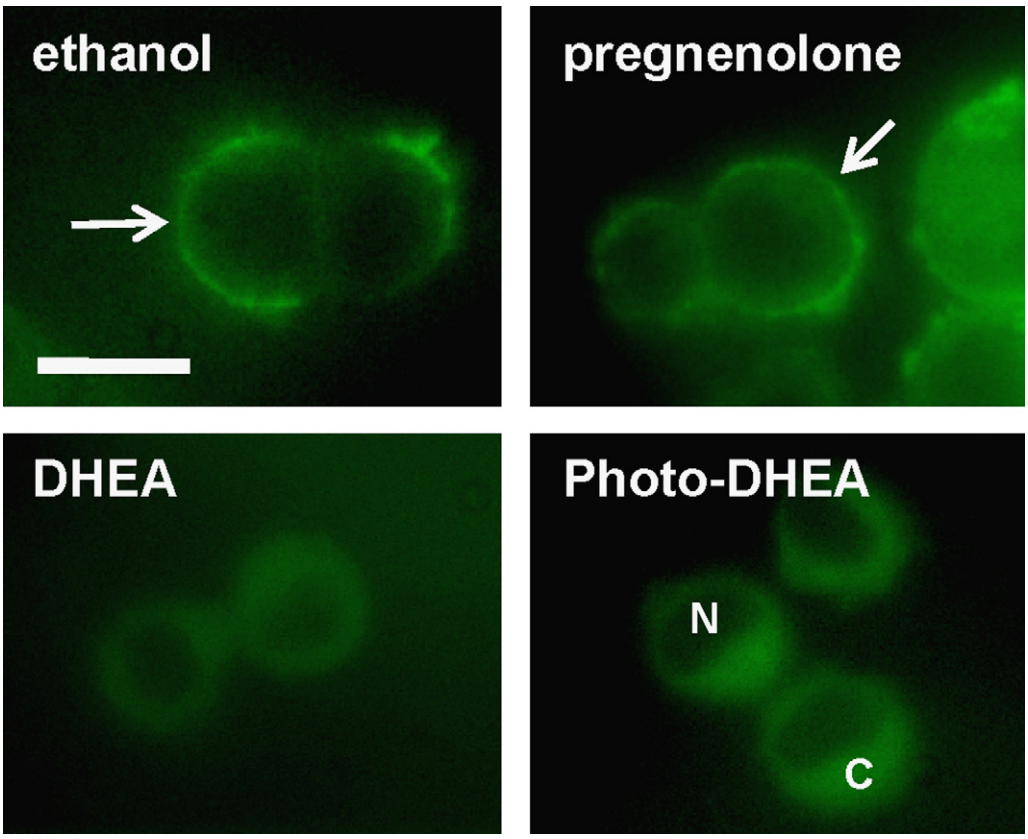


Fig. 2. Photo-DHEA competes with DHEA-Bodipy (green) for DHEA binding-sites on the plasma membrane of neuronal PC12 cells. PC12 cells were incubated simultaneously with 1 μM DHEA-Bodipy ± 30 μM steroid as indicated. Pictures of DHEA-Bodipy were taken 2 min after addition of the steroids. The scale bar represents 10 μm. Arrow, plasma membrane; N, nucleus; C, cytoplasm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Ø 2.5 cm) with ethylacetate and rechromatographed with ethylacetate/aceton (3/2) to afford 290.8 mg (0.50 mmol, 95%) of **2** as colorless solid.

^{1}H NMR ($CDCl_3$, 300 MHz), δ [ppm]: 0.86 (s, 3H, C_{18}-methyl), 1.14 (s, 3H, C_{19}-methyl), 1.45–2.61 (m, 18H, remaining C–H of the steran), 3.52–3.68 (m, 6H, –NH–CH_2–CH_2–NH–), 4.54 (s, 2H, CH_2-Oximino), 6.49 (m, 1H, C_6–H), 6.52 (d, 1H, ar, J = 2.1 Hz), 6.59 (d, 1H, ar, J = 2.4 Hz), 6.81–6.91 (m, 1H, –NH–), 7.44–7.47 (d, 1H, ar, J = 8.4 Hz), 7.83–7.90 (m, 1H, –NH–).

^{13}C NMR (75.5 MHz) δ 220.72, 174.39, 172.70, 170.13, 170.10, 162.82, 162.74, 158.25, 158.23, 156.40, 145.66, 128.00, 112.53, 111.13, 111.12, 111.10, 111.08, 111.06, 110.02, 109.91, 107.80, 77.48, 77.05, 76.63, 72.57, 70.63, 53.77, 49.82, 47.85, 47.83, 45.96, 42.12, 40.97, 39.08, 38.52, 37.13, 36.61, 36.44, 35.30, 31.52, 31.00, 30.54, 29.18, 25.38, 24.87, 20.75, 20.07, 17.97, 13.88, 13.83, 13.81.

ESI MS m/z: 601.30 $[M+Na]^+$, 1179.52 $[2M+Na]^+$.

TLC: detection UV and ninhydrin (brown spot). R_f: 0.3 (ethylacetate), 0.6 (ethylacetate/acetone (3:2, v/v)).

HPLC elution profile: see Supporting Information.

To determine the time course of the photolysis of Photo-DHEA, 8.5 μg of Photo-DHEA in 1 ml of ethanol/methanol (1/32) were irradiated at λ > 335 nm using a mercury arc lamp (200 W). When the absorbance at λ_{max} = 265 nm was monitored, it decreased by 50% only 20 s after UV irradiation. After 3 min the absorbance became almost equivalent to the basal level (see Supporting Information).

2.3. Biology

2.3.1. Cell culture

PC12 rat pheochromocytoma cells were cultured on poly-L-lysine coated cell culture dishes in phenol red-free RPMI medium 1640 containing 10% horse serum, 5% fetal calf serum (FCS), 2 mM glutamine, and penicillin/streptomycin. HepG2 cells were grown in phenol red-free DMEM high glucose supplemented with 10% FCS, 2 mM glutamine, and penicillin/streptomycin. All cell lines were cultured at 37 °C, 5% CO_2, and 100% humidity.

2.3.2. Live-cell imaging of PC12 cells

For live-cell imaging, PC12 cells were seeded on 18 mm coverslips coated with poly-L-lysine plus collagen in 3.5 cm cell culture dishes. 24 h before the experiment, cells were cultivated in phenol red-free medium containing charcoal-stripped FCS, glutamine, and penicillin/streptomycin. For live cell imaging, the coverslip was mounted on a thermostated chamber (37 °C) on an Axiovert S100 microscope (Zeiss). For displacement experiments, PC12 cells were incubated with 1 μM DHEA-Bodipy ± 30 μM pregnenolone, DHEA or Photo-DHEA. Pictures were taken at an excitation wavelength of λ = 490 nm for DHEA-Bodipy. Fluorescence emission was measured using a Q505LP filter. Cells were always checked for autofluorescence.

2.3.3. GloSensor™ cAMP assay

For the GloSensor™ cAMP assay (Promega), HepG2 cells were seeded on 96 well-plates coated with poly-L-lysine in culture medium without antibiotics. At 90% confluency, cells were transiently transfected with pGloSensor™-20F cAMP plasmid. In brief, 100 ng (0.1 μl) plasmid was added to 50 μl serum-free culture medium per well. 1 μl Lipofectamine™ 2000 (Invitrogen) was added to 50 μl serum-free culture medium. Diluted plasmid and diluted Lipofectamine™ were combined and incubated at room temperature for 20 min. Cells were

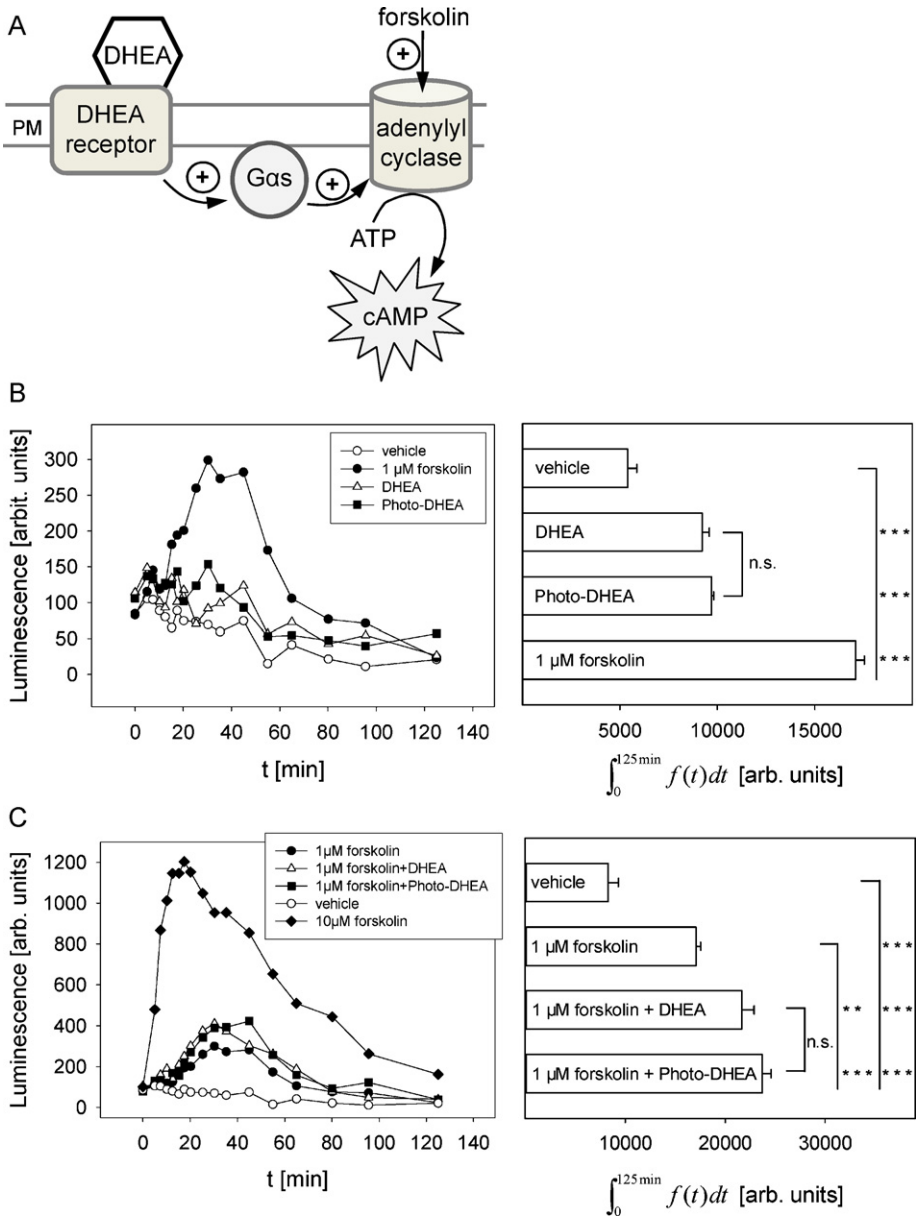


Fig. 3. (A) In HepG2 liver cells, DHEA binds to and activates a putative G protein-coupled receptor which in turn activates an adenylyl cyclase via the Gαs subunit of a heterotrimeric G protein. The adenylyl cyclase synthesizes cAMP which can be monitored by a GloSensor™ cAMP assay (luminescence in (B) and (C)). The plant diterpene forskolin is a direct activator of adenylyl cyclases. (B, left) Both, 30 μM DHEA and 30 μM Photo-DHEA induce the biosynthesis of the second messenger cAMP in human HepG2 liver cells. (C, left) The effect of DHEA and Photo-DHEA is additive to the partial stimulation of the adenylyl cyclase by 1 μM of forskolin. Values correspond to the mean of three samples. (B and C, right) For each treatment, areas below the curves of three samples were calculated and expressed as the mean ± SD. Statistical differences between the mean values were determined by One Way-ANOVA/Bonferroni *t*-test. (***$P < 0.001$: vehicle versus 1 μM of forskolin, DHEA ± 1 μM of forskolin, and Photo-DHEA ± 1 μM of forskolin, respectively; **$P < 0.01$: 1 μM of forskolin versus 1 μM of forskolin + DHEA, ***$P < 0.001$: 1 μM of forskolin versus 1 μM of forskolin + Photo-DHEA; n.s.: not significant). Vehicle: DMSO (0.01%) + ethanol (0.1%). PM: plasma membrane.

incubated with the DNA/transfection complex (100 μl/well) at 37 °C in a CO_2 incubator. 5 h after transfection, medium was replaced by 100 μl phenol red-free medium containing charcoal-stripped FCS, glutamine, and penicillin/streptomycin. Cells were incubated overnight at 37 °C in a CO_2 incubator. Then, cells were incubated in 90 μl/well of equilibration medium (DMEM, 2 mM glutamine, 10 mM Hepes (pH 7.4), 10% charcoal-stripped FCS, 2% GloSensor™ cAMP Reagent) at room temperature for 2 h. 10 μl of the compound of interest in medium (DMEM, 2 mM glutamine, 10 mM Hepes (pH 7.4), 10% charcoal-stripped FCS) was added and luminescence was measured repeatedly from individual wells in a FLUOstar OPTIMA microplate reader (BMG LABTECH GmbH) to record the kinetic response.

3. Results and discussion

To determine whether Photo-DHEA is a functional photoreactive DHEA analog, two cell-based assays were employed. (i) Displacement of a functional fluorescent DHEA-derivative (DHEA-Bodipy) from rat pheochromocytoma (PC12) cells and (ii) induction of cyclic adenosine monophosphate (cAMP) synthesis in human liver (HepG2) cells.

3.1. Photo-DHEA and DHEA bind rapidly to the plasma membrane of PC12 cells

DHEA has been reported to protect neuronal PC12 cells from programmed cell death (apoptosis) which normally occurs upon

serum withdrawal [14]. DHEA exerts this neuroprotective effect upon binding to specific plasma membrane binding sites [15]. Recently, we introduced DHEA-Bodipy, a functional fluorescent DHEA-derivative suitable for live cell imaging of intracellular DHEA transport and localization [7]. DHEA-Bodipy binds rapidly (within 2 min) and specifically to the plasma membrane of living PC12 cells as could be shown by co-localization experiments with the plasma membrane marker CellMask™ Deep Red and displacement experiments with different steroids [7]. Here, we demonstrate that both, Photo-DHEA and DHEA (5-androsten-3β-ol-17-one), compete with DHEA-Bodipy for DHEA-binding sites at the plasma membrane of PC12 cells (Fig. 2). This suggests a direct interaction between Photo-DHEA and the putative DHEA receptor. In contrast, pregnenolone (5-pregnen-3β-ol-20-one), which differs from DHEA at position C17, does not compete with DHEA-Bodipy for binding to PC12 cells (Fig. 2).

PC12 cells have been demonstrated to possess saturable high-affinity DHEA binding sites [15]. The C17-keto group of DHEA interacts specifically with the putative DHEA receptor whereas the C3-β-hydroxy function is not necessary for plasma membrane binding of DHEA in these cells [7,15]. Recently, Olivo et al. developed a derivative of DHEA with both a photoreactive benzophenone group and a biotin group at position C17 [16]. Although this DHEA derivative lacks the 17-keto function of DHEA it specifically labeled plasma membranes of bovine aortic endothelial cells (BAEC) [17]. In contrast to PC12 cells, DHEA binding sites of BAEC tolerate large modifications at position C17 of DHEA whereas the hydroxyl function at position C3 of DHEA is mandatory [18]. This indicates that there might be species and/or cell type specific receptor subtypes for DHEA or, in general, multiple DHEA receptors from different protein families. Sex hormones differ mainly at positions C3 and C17, underlining the importance to leave these positions unchanged when developing fluorescent or photoreactive steroid analogs. We chose DHEA-7-CMO for derivatization with the photoreactive aryl azido group in order to retain an unaltered DHEA core structure with a long spacer as a flexible link between the steroid hormone and the photoreactive group. Therefore, Photo-DHEA introduced in this work should be able to label DHEA receptors with very stringent requirements for the DHEA structure.

3.2. Photo-DHEA and DHEA induce cAMP synthesis in HepG2 cells

DHEA has been reported to induce the synthesis of the second messenger cAMP in chicken liver cells [19]. DHEA might bind to and activate a putative G protein-coupled receptor in the plasma membrane of these cells which in turn activates a heterotrimeric G protein. The Gαs subunit of the heterotrimeric G protein will then stimulate the enzyme adenylyl cyclase which catalyses the production of cAMP from ATP (Fig. 3A). In the downstream signaling cascade, cAMP activates the protein kinase A (PKA) which phosphorylates and inactivates the liver X receptor (LXR) [20]. As a consequence, the DNA-binding of LXR is suppressed. This decreases the expression of SREBP-1, a transcription factor for fatty acid and triacylglycerol biosynthesis. The resulting decrease in fatty acid and triacylglycerol biosynthesis might contribute to the beneficial effects of DHEA on obesity [21]. Using the GloSensor™ cAMP assay, we found that both Photo-DHEA and DHEA stimulated the adenylyl cyclase of human HepG2 liver cells (Fig. 3B, left). Moreover, we found that the stimulation by DHEA or Photo-DHEA is additive to the partial stimulation of the adelylyl cylase with 1 μM of the plant diterpene forskolin, a direct activator of adenylyl cyclases (Fig. 3C, left). Calculation of the areas below the curves and application of One Way-ANOVA/Bonferroni *t*-test show that these effects are statistically significant (Fig. 3B and C, right). These results demonstrate that both DHEA and Photo-DHEA activate the adenylyl cyclase in liver cells probably via a G protein-coupled pathway.

4. Conclusion

In spite of the numerous results concerning the mechanisms of DHEA action, it is still a matter of debate whether there is a single, specific receptor for DHEA or whether the biological actions of DHEA involve multiple receptors [3,22]. Using a neuronal and a hepatic cell line, we have shown here that Photo-DHEA is a cell biologically active DHEA derivative. Radioiodination of Photo-DHEA at position C5 of the photoreactive group 4-azidosalicylic acid [23] could be achieved by incubation of Photo-DHEA with Na[^{125}I] in the presence of the iodination reagent IODO-GEN (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) which can be coated onto a reaction vessel as a solid-phase oxidant [24]. Radioactive iodination of Photo-DHEA should yield a valuable photoreactive DHEA analog suitable for photoaffinity labeling studies for detection and identification of the elusive DHEA receptor(s). Receptor identification is crucial for our understanding of the plethora of physiological and pathophysiological mechanisms DHEA is involved in.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.01.009.

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