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Phosphorylation and function of the hamster adrenal steroidogenic acute regulatory protein (StAR)

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Abstract

In order to study the effect of phosphorylation on the function of the steroidogenic acute regulatory protein (StAR), 10 putative phosphorylation sites were mutated in the hamster StAR. In pcDNA3.1-StAR transfected COS-1 cells, decreases in basal activity were found for the mutants S55A, S185A and S194A. Substitution of S185 by D or E to mimic phosphorylation resulted in decreased activity for all mutants; we concluded that S185 was not a phosphorylation site and we hypothesized that mutations on S185 created StAR conformational changes resulting in a decrease in its binding affinity for cholesterol. In contrast, the mutation S194D resulted in an increase in StAR activity. We have calculated the relative rate of pregnenolone formation (App. V_{max}) in transfected COS-1 cells with wild type (WT) and mutant StAR-pcDNA3.1 under control and $(Bu)_2$-cAMP stimulation. The App. V_{max} values refer to the rate of cholesterol transported and metabolized by the cytochrome P450scc enzyme present in the inner mitochondrial membrane. The App. V_{max} was 1.61 ± 0.28 for control (Ctr) WT StAR and this value was significantly increased to 4.72 ± 0.09 for $(Bu)_2$-cAMP stimulated preparations. App. V_{max} of 5.53 (Ctr) and 4.82 ($(Bu)_2$-cAMP) found for S194D StAR preparations were similar to that of the WT StAR stimulated preparations. At equal StAR quantity, an anti-phospho-(S/T) PKA substrate antibody revealed four times more phospho-(S/T) in $(Bu)_2$-cAMP than in control preparations. The intensity of phosphorylated bands was decreased for the S55A, S56A and S194A mutants and it was completely abolished for the S55A/S56A/S194A mutant. StAR activity of control and stimulated preparations were diminished by 73 and 72% for the mutant S194A compared to 77 and 83% for the mutant S55A/S56A/S194A. The remaining activity appears to be independent of phosphorylation at PKA sites and could be due to the intrinsic activity of non-phosphorylated StAR or to an artefact due to the pharmacological quantity of StAR expressed in COS-1. In conclusion we have shown that $(Bu)_2$-cAMP provokes an augmentation of both the quantity and activity of StAR, and that an enhancement in StAR phosphorylation increases its activity. The increased quantity of StAR upon $(Bu)_2$-cAMP stimulation could be due to an augmentation of its mRNA or protein synthesis stability, or both; this is yet to be determined.

Keywords: Hamster; Adrenal; ACTH; Angiotensin; TPA; StAR; Cyclic AMP; Phosphorylation; 2D-PAGE

1. Introduction

The rate limiting step of steroidogenesis is the delivery of cholesterol to the matrix side of inner mitochondria membrane where resides the cholesterol side-chain cleavage cytochrome P450 (P450scc), the enzyme catalyzing the transformation of cholesterol to pregnenolone [1]. The transfer of cholesterol into mitochondria across the intermembrane aqueous space and steroidogenesis are both inhibited by the protein synthesis inhibitor cycloheximide [2,3].

Abbreviations: StAR, steroidogenic acute regulatory protein; $(Bu)_2$-cAMP, dibutyryl cAMP; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; P450scc, cytochrome P450 side-chain cleavage; PKA, cAMP-dependent protein kinase; PKC, calcium-dependent protein kinase; A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; R, arginine; S, serine; T, threonine

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In lipoid congenital adrenal hyperplasia, mutations in the steroidogenic acute regulatory protein (StAR) gene were shown to be responsible for the defective steroidogenesis [4–8]. In vitro, the expression of StAR was shown to induce steroidogenesis without hormonal stimulation [1]. Furthermore, impaired steroidogenesis and lipid accumulation in steroidogenic tissues were obtained in StAR gene nullizygous mice produced by homologous recombination [9]. This genetic and biochemical evidence has indicted StAR as a key molecule in regulating cholesterol transfer across the mitochondria membrane and consequently in controlling the transformation of cholesterol to pregnenolone.

Up to now, three models have been proposed to describe the mechanism of action of StAR on the cholesterol entry into mitochondria. One model suggests cholesterol desorption from the outer side of the outer membrane to the inner membrane [10], the second model proposes that StAR is active as a molten globule [11] and in the third model, cholesterol is transferred via an intermembrane shuttle mechanism [12].

Two main factors regulate steroidogenesis in the adrenal cortex, adrenocorticotropin hormone (ACTH) and angiotensin II (AII), both regulating steroid formation at the site of transformation of cholesterol to pregnenolone [13]. We have demonstrated that ACTH administration [14,15] and sodium restriction [16] in vivo affected the formation of different species of StAR in rat [14,16,17] and hamster [14] adrenals.

The labelling of phosphoproteins upon ACTH and gonadotropin treatment of steroidogenic cells were reported well before their identification in StAR [2,18–23], and also recently using ^{35}S-methionine or ^{32}P-orthophosphate; Arakane et al. [24] showed that ACTH and AII affected the labelling of 30 kDa phosphoproteins in steroidogenic cells. Moreover, using COS-1 cells to express human StAR, the mutation of a potential protein kinase A-mediated phosphorylation site at serine 195 to alanine (S195A) reduced ^{32}P incorporation from labeled ATP into StAR. The capacity of that mutant to induce pregnenolone production was also reduced by 50%, indicating the importance of phosphorylation as part of the human StAR mechanism of action to control steroidogenesis. Up to now, the remaining capacity of transfected COS-1 cells by the S195A to synthesize pregnenolone has not been attributed to other phosphorylation sites and no systematic studies have been completed to cover this subject.

Since the original report on the mouse StAR [1], StAR was cloned from 13 other animal species including the hamster [15]. By alignment analysis of the amino acid sequences of all these StARs, we have found conserved putative phosphorylation sites among most animal species. In order to determine if these putative phosphorylation sites were part of the mechanism of action of StAR, we have mutated putative phosphorylation sequences in the hamster StAR and we have studied their effects in transfected COS-1 cells on pregnenolone synthesis, and on StAR phosphorylation. We found that S55, S56 and S194 are phosphorylation sites in the hamster StAR and that phosphorylation of these sites has in impact on StAR activity. Results of this study have been communicated in part at the Endocrine Society's 84th meeting held in San Francisco 19–22 June 2002, and appeared in abstract P1-497, page 269 of the Program and Abstracts book.

2. Materials and methods

2.1. Materials

All restriction and modifying enzymes were purchased from New England Biolabs (Mississauga, ON, Canada). The expression vector pcDNA3.1-Myc-His, Lipofectamine, pancreatin, oligonucleotides and the anti-myc antibody (mouse) were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). All vectors were amplified in *E. coli* XL1-Blue competent cells (Stratagene, La Jolla, CA), and purified on anion-exchange columns (Qiagen, Chatsworth, CA). TPA and $(Bu)_2$-cAMP were obtained from Sigma–Aldrich (St.-Louis, MO). The anti-phospho-(S/T) PKA substrate antibodies (rabbit) were purchased from Cell Signaling Technology (Beverly, MA). The PWO DNA polymerase was bought from Roche Diagnostics (Laval, QC, Canada).

2.2. Mutagenesis

StAR sequences of man [25], horse [26], pig [27], sheep [28], cow [29], rat [30], mouse [1], hamster [15], chicken [31], frog [31], zebra fish [31], rainbow trout [32], brook trout [32], eel [33] and cod [34] were obtained from Genbank and aligned with ClustalW at http://www.ebi.ac.uk. Putative phosphorylation sites were determined using PhosphoBase v2.0, a database of phosphorylation sites provided by the Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/databases/PhosphoBase). All mutations were generated by PCR [35]. WT-StAR and all mutants were cloned into pcDNA3.1-Myc-His and entirely sequenced between used restriction sites using T7 sequencing kit from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada), amplified, and purified on Qiagen anion-exchange columns.

2.3. Hamster StAR expression

Either WT or mutated StAR was transiently transfected into monkey kidney COS-1 cells (American Type Culture Collection, Rockville, MD) using the lipofectamine method. Twenty-four hours before transfection, the cells were harvested with pancreatin-EDTA solution and plated at an initial density of 3.5×10^5 cells per 10 cm^2 well in six-well plates. Cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS), 5.96 μg/L HEPES, 2.2 g/L $NaHCO_3$, 1 μm L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulphate. DMEM containing no FBS and no antibiotics was used for transfections. Each transfection assay

received 1 μg of DNA (500 ng F2 construct [36] containing P450scc-adrenodoxin reductase-adrenodoxin and 500 ng of pcDNA3.1 + pcDNA3.1-StAR) and 10 μg lipofectamine in one ml of media. Twenty-two hours after transfection cells were rinsed with PBS. Cells were then incubated for 24 h in DMEM without phenol red containing 10% dextran-coated charcoal-treated FBS and antibiotics with or without stimulating agents. Media were kept for pregnenolone quantification and cells for immunoblotting analyses.

2.4. Measurement of the hamster StAR activity

In this study, StAR activity is defined as the quantity of pregnenolone formed by co-transfected COS-1 cells with F2 and StAR plasmids. Pregnenolone formation was analyzed by radioimmunoassay (ICN Pharmaceuticals, Diagnostics Division, Orangeburg, NY).

2.5. Immunoblotting analysis

Cells were scraped and directly harvested in hot (100 °C) Laemmli buffer [37]. All samples were then passed through a 26 gauge needle, then boiled for 10 min and finally centrifuged at 12,000 × *g* for 2 min. Protein levels were determined on the supernatant using Bio-Rad's Protein Assay Dye Reagent (Mississauga, ON, Canada). For mitochondrial preparations, cells were suspended in the homogenization buffer and sonicated for 7 s. The degree of cell rupture was verified under microscope. When totally ruptured, the cell preparation was centrifuged 10 min at 900 × *g* and only the supernatant was kept and centrifuged at 15 min at 9500 × *g*. The mitochondria pellet was resuspended in 75 μl homogenization buffer and 25 μl Laemmli buffer (4×) [37].

Soluble proteins were separated by SDS-PAGE and analyzed by immunoblotting as previously described [38,39] using a rabbit polyclonal anti-mouse StAR antibody [40]. Immunoreactive proteins were detected using ECL-PLUS light emitting reagents (Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK), and quantified on STORM 860 using ImageQuant software version 5.0 (Molecular Dynamics, Sunnyvale, CA).

2.6. Statistical analyses

Differences between experimental groups were analyzed by one way ANOVA using the SigmaStat program for Windows (SPSS Science, Chicago, IL).

3. Results

3.1. Pregnenolone production by COS-1 cells transfected with different StAR plasmids

A strong amino acid sequence identity exists between StAR indexed from 13 different vertebrate species. These StARs could undergo post-translational changes, which can modify protein activity. Indeed, StAR contains putative phosphorylation sites for PKA, PKC, and casein kinase 2 (CK2). StAR amino acid sequences were thus analyzed in silico to determine putative phosphorylation sites conserved among species. Out of 19 putative sites discovered, nine of them were shown to be conserved among most mammal species including the hamster (Table 1). The hamster StAR S55, S56, S60, S68, S90, S185, S194, S276 and T262 putative phosphorylation sites were mutated; T was changed to valine (V) and S to alanine (A), respectively, to systematically study their roles in StAR activity. The S270A mutant was also made although this putative phosphorylation site was less conserved among species.

To test StAR activity, COS-1 cells were co-transfected with the expression plasmid F2 (harbouring P450scc-adrenodoxin reductase-adrenodoxin) and pcDNA3.1-StAR. Fig. 1 shows the relative enhancing effect of increasing concentrations of the hamster wild type (WT) StAR DNA in transfection media on StAR activity. In this series of experiments, basal activity of cells co-transfected with the empty pcDNA3.1 vector and F2 was 21.3 ng pregnenolone/3.5 × 10^5 cells, and this activity was increased in the presence of pcDNA3.1-WT-StAR. For control, with 50 ng of StAR DNA used for treansfection, the production of pregnenolone was 72 ng/3.5 × 10^5 cells. Compared to control, with 50 ng of StAR DNA used for transfections, $(Bu)_2$-cAMP stimulated the production of pregnenolone by a factor of four. In contrast, the addition of TPA in the incubation media of transfected COS-1 cells with WT-pcDNA3.1 appeared to have no enhancing effect on basal StAR activity (results not shown).

3.2. Kinetic studies

Kinetic analyses were performed to determine if $(Bu)_2$-cAMP affected StAR activity. We have determined the rate of the reaction (App. V_{max}—reference to the rate of cholesterol transported and metabolized by the cytochrome P450scc enzyme present in the inner mitochondrial membrane) and the apparent Michaelis–Menten constant (App. K_M). For standardization between results from different experiments, the value 1 was arbitrarily attributed to the quantity of StAR measured in COS-1 cells transfected with 50 ng of WT StAR DNA and to the quantity of pregnenolone formed in the corresponding incubation media. Figs. 2 and 3 show results of experiments performed on WT control and $(Bu)_2$-cAMP stimulated preparations for immunoblotting and kinetic analyses, respectively (for statistical analysis see Table 2). Table 2 summarizes results calculated separately from 5 WT control and 3 WT $(Bu)_2$-cAMP different experiments. The App. V_{max} and App. V_{max}/App. K_M ratio values were statistically different ($P < 0.05$) between WT control and WT $(Bu)_2$-cAMP preparations. These results show that StAR preparations stimulated by $(Bu)_2$-cAMP were 2.9 times more active than the controls. This suggests that the stimulation of StAR by endogenous cAMP was weaker than that provoked

Table 1
Putative phosphorylation sites in StAR from various animal species

Position	S55/S56	S56/S57	S60/S61	S68/S69	S90/S91	S185/S186	S194/S195	T262/T263	S276/S277
Hamster	+	+	+	+	+	+	+	+	+
Mouse	+	+	+	+	–	+	+	+	–
Rat	+	+	+	+	–	+	+	+	+
Human	+	+	+	+	+	+	+	+	+
Porcine	+	+	+	+	+	–	+	+	+
Bovine	–	+	+	+	–	+	+	+	+
Ovine	–	+	+	+	–	+	+	+	+
Horse	+	+	+	+	–	+	+	+	+
Chicken	+	+	+	+	–	+	+	+	-
Frog	–	+	+	–	–	+	+	+	+
Rainbow trout	+	–	+	–	–	+	+	+	–
Brook trout	+	+	–	–	–	+	+	+	–
Zebra fish	+	–	–	–	–	–	T194	–	–

StAR sequences were obtained from Genbank. Putative phosphorylation sites were determined using PhosphoBase v2.0, a database of phosphorylation sites provided by Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/databases/PhosphoBase). PKA, protein kinase A; PKC, protein kinase C; CK2, casein kinase II; –, absence and +, presence of a phosphorylation consensus sequence.

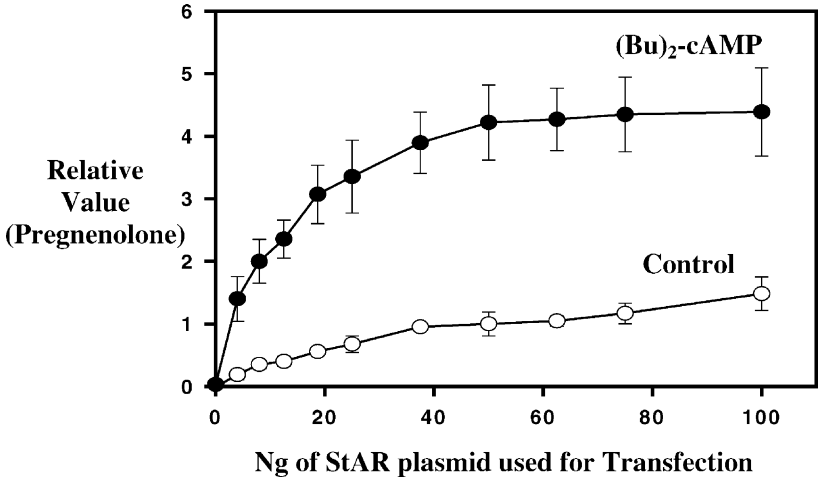

Fig. 1. Pregnenolone production by COS-1 cells transfected with different quantities of StAR plasmid. COS-1 cells co-transfected with the F2 construct (500 ng) and increasing concentrations of WT StAR-pcDNA3.1 and pcDNA3.1 (to a total of 1000 ng of DNA), were incubated without (control) or with $(Bu)_2$-cAMP (1 mM) for 24 h. Pregnenolone was analyzed by RIA. Results are mean ± S.E.M. of values obtained from three different preparations performed in triplicate. In this series of experiments, pregnenolone values found in COS-1 cells transfected with F2 and pcDNA3.1 plasmids were subtracted from all experimental values. The relative value of 1 was arbitrarily attributed to pregnenolone formed by control preparations of COS-1 cells transfected with 50 ng of WT StAR-pcDNA3.1, which corresponds to 72 ng of pregnenolone formed by 3×10^5 cells in 2.5 ml.

by the addition of a high $(Bu)_2$-cAMP concentration. Moreover, in this series of experiments we evaluated that at equal quantity of StAR (take for example transfection made with 18.7 ng of WT StAR for $(Bu)_2$-cAMP versus 67–75 ng for control (Fig. 2)) the StAR activity was 2.3 times more active (Fig. 1) in $(Bu)_2$-cAMP than in control preparations.

3.3. Mutants

In preliminary experiments, COS-1 cells were co-transfected with the F2 construct and either the WT-StAR or mutated-StAR plasmids. Compared to WT, basal StAR activity was not affected for mutants S56A, S60A, S68A, S90A,

Table 2
Comparison of kinetics parameters between WT and S194D mutants

Preparation	App. V_{max} (mean ± S.E.M)	App. K_M (mean ± S.E.M.)	App. V_{max}/App. K_M (mean ± S.E.M.)	*n*
WT Ctr	1.61 ± 0.28	0.68 ± 0.22	2.79 ± 0.35	5
WT $(Bu)_2$-cAMP	4.72 ± 0.91	0.77 ± 0.22	7.53 ± 1.82	3
S194D Ctr	5.53 ± 1.06	1.08 ± 0.35	6.25 ± 1.80	3
S194D $(Bu)_2$-cAMP	4.82 ± 0.67	0.78 ± 0.13	6.90 ± 2.24	3

COS-1 cells co-transfected with the F2 construct (500 ng) and increasing concentrations of WT or S194D StAR-pcDNA3.1 and pcDNA3.1 (to a total of 1 μg of plasmids), were incubated without (control, Ctr) or with $(Bu)_2$-cAMP (1 mM) for 24 h. Pregnenolone was analyzed by RIA; StAR was analyzed by immunoblotting and quantified on a PhosphorImager. Each experiments was performed in duplicate. Number of experiments performed in duplicate, *n*.

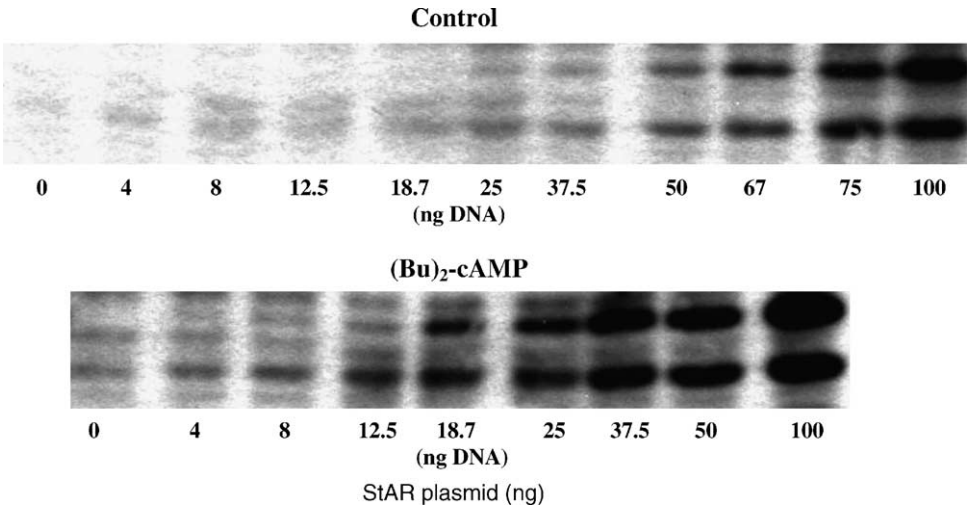

Fig. 2. StAR protein levels produced by COS-1 cells transfected with different quantities of StAR plasmid. COS-1 cells co-transfected with the F2 construct (500 ng) and increasing concentrations of WT StAR-pcDNA3.1 and pcDNA3.1 (to a total of 1000 ng of DNA), were incubated without (control) or with $(Bu)_2$-cAMP (1 μm) for 24 h. StAR was analyzed by immunoblotting. Data shown are from three different experiments performed in duplicate (for statistics see Table 2).

T262V, S270A and S276A. However, StAR activity was significantly decreased by the S55A, S185A and S194A mutants (results not shown). When $(Bu)_2$-cAMP was added to the incubation media, the activity of all preparations was increased; increases observed for the mutants were comparable to that of WT except for mutants S185A and S194A. Interestingly, the $(Bu)_2$-cAMP stimulated activity of the S55A mutant was comparable to that of the WT, suggesting a rather weak participation of this site in controlling StAR activity. Compared to their own control, the presence of TPA in incubation media did not change StAR activity of any preparation (results not shown).

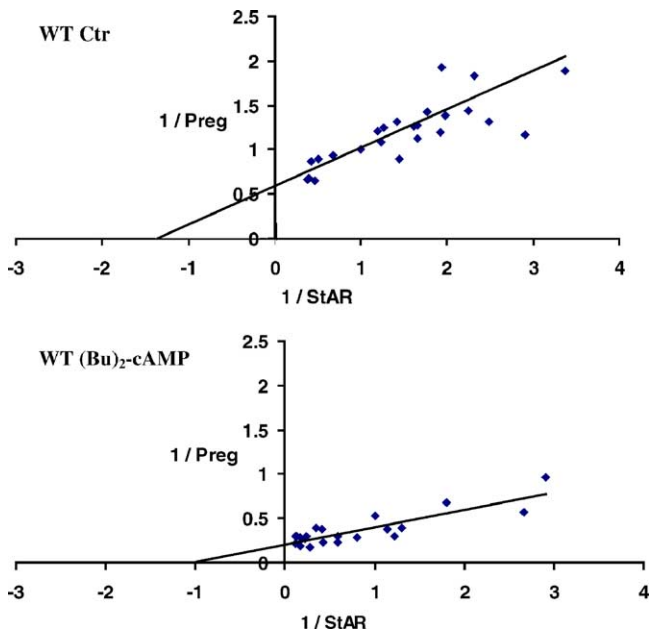

Fig. 3. Kinetic studies. COS-1 cells co-transfected with the F2 construct (500 ng) and increasing concentrations of WT StAR-pcDNA3.1 and pcDNA3.1 (to a total of 1 μg of DNA), were incubated without (Control, Ctr) or with $(Bu)_2$-cAMP (1 μm) for 24 h. Pregnenolone was analyzed by RIA; StAR was analyzed by immunoblotting. Data shown are from three different experiments performed in duplicate (for statistics see Table 2).

Nothing is known about the putative phosphorylation site S185 in the hamster, or the equivalent conserved S186 in human. To further study if the hamster S185 could effectively be phosphorylated or not, we have made three additional mutations. We have substituted the serine to the charged glutamic acid (E) or aspartic acid (D) residues in order to mimic phosphorylation. We have also substituted the serine into a cysteine (C), effectively replacing the serine OH group by a SH group. The SH group has similar properties to the OH group but cannot be phosphorylated. Compared to WT, S185E and S185D mutants had a decreased basal StAR activity similar to that of the S185A mutant (Fig. 4A). In addition, the activity of these two mutants was not stimulated by $(Bu)_2$-cAMP. This indicates that S185 is not a phosphorylation site. In contrast to these, the substitution S185C resulted in a partial recovery of basal StAR activity; and this activity was stimulated by $(Bu)_2$-cAMP; this further indicates that S185 is not a phosphorylation site. As analyzed by western blotting, the respective basal and $(Bu)_2$-cAMP stimulated StAR expression levels were not decreased by the mutations S185A, S185E, S185C (Fig. 4B and C) and S185D (not shown). This indicates that the change in StAR activity by these mutants was due to the amino acid substitutions and not to differences in protein levels. Thus, taken together these results clearly demonstrate that S185 is not a phosphorylation site for the hamster StAR.

The hamster StAR amino acid residue S194 corresponds to the conserved S195 in human StAR that Arakane et al. [24] have reported to be phosphorylated. As mentioned earlier, the

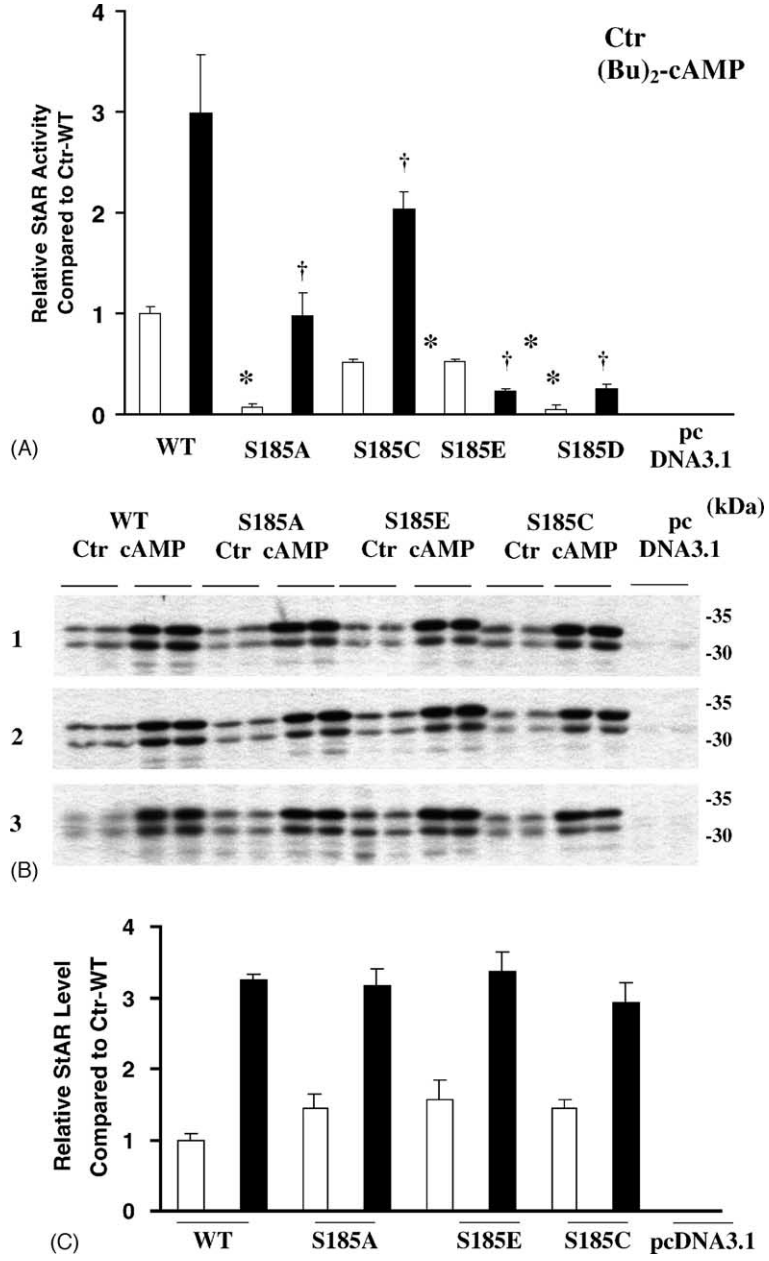

Fig. 4. Phosphorylation simulation on S185 of the hamster StAR. COS-1 cells co-transfected with the F2 construct (500 ng), different StAR-pcDNA3.1 plasmids (200 ng) and pcDNA3.1 (300 ng) were incubated without (control, Ctr) or with ($(Bu)_2$-cAMP, cAMP) (1 μm) for 24 h. (A) Relative StAR activity; the pcDNA3.1; the relative value of 1 was arbitrarily attributed to pregnenolone formed by control preparations of COS-1 cells transfected with 200 ng of WT StAR-pcDNA3.1, which corresponds to 125 ng of pregnenolone formed by 3×10^5 cells in 2.5 ml. Values are the mean ± S.E.M. of three different experiments performed in duplicate. $^*P < 0.05$ compared to control, $^\dagger P < 0.05$ compared to stimulated WT preparations, respectively. (B) Immunoblotting was done in duplicate on three different total COS-1 cell preparations (20 μg proteins) using an anti-StAR antibody. (C) Relative StAR level compared to control.

hamster mutant S194A had a lower basal StAR activity than the WT, and to further study this we have engineered the S194C and the S194D mutants. The activity of the mutant S194C was similar to that of mutant S194A in the absence or presence of $(Bu)_2$-cAMP, respectively (results not shown). However, as shown in Fig. 5, the mutation S194D had an enhancing effect on StAR activity. Indeed, in control preparations (with 50 ng of StAR used for transfection), the S194D mutant was twice more active than its WT counterpart. Interestingly, the addition of $(Bu)_2$-cAMP in incubation media further stimulated StAR activity. This could be due to a greater stability conferred by the phosphorylation of other putative sites or by other undefined mechanisms. Table 2 shows that App. V_{max} and App. V_{max}/App. K_M values of S194D were similar to that of WT $(Bu)_2$-cAMP stimulated preparations. This indicates that a change in amino acid residue at position 194 by a negatively charged amino acid residue was able to enhance StAR activity.

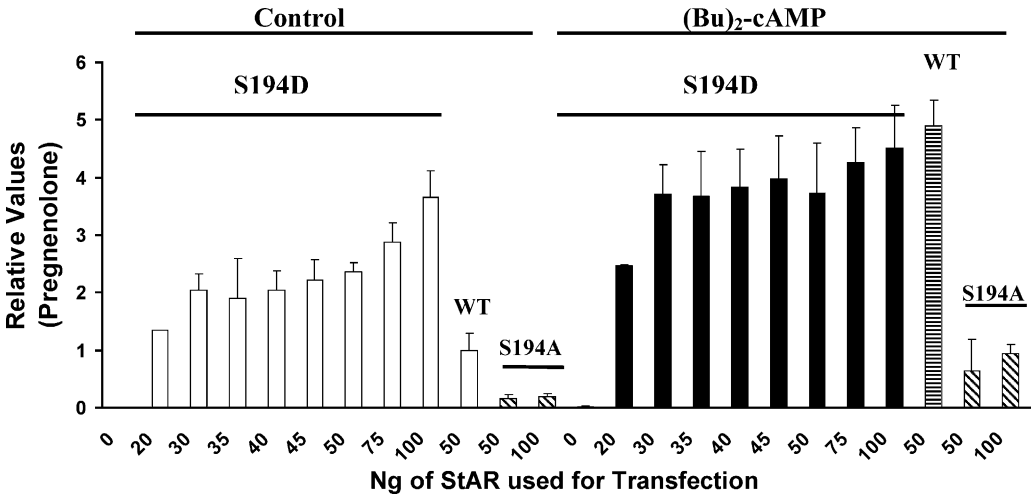

Fig. 5. Effects of $(Bu)_2$-cAMP on S194A and S194D StAR mutants. COS-1 cells co-transfected with the F2 construct (500 ng) and increasing concentrations of S194D StAR-pcDNA3.1 and pcDNA3.1 (to a total of 1 μg of DNA), were incubated without (control) or with $(Bu)_2$-cAMP (1 μm) for 24 h. S194A and WT StAR-pcDNA3.1 were also used for transfection at indicated concentrations. Pregnenolone was analyzed by RIA. The relative value of 1 was arbitrarily attributed to pregnenolone formed for control preparations of COS-1 cells transfected with 50 ng of WT StAR-pcDNA3.1 which corresponds to 68 ng (background subtracted) of pregnenolone formed by 3×10^5 cells in 2.5 ml. Results are expressed as mean ± S.E.M. of three different experiments performed in duplicate.

3.4. Immunoblotting analyses using an anti-phospho-(S/T) PKA substrate antibody

We found that a specific anti-phospho-(S/T) PKA substrate antibody could be used for immunoblotting studies on mitochondrial StAR preparations solubilized in Laemmli buffer. At equal quantity of StAR (Fig. 6A), four-times less phosphorylated proteins were detected by the anti-phospho-(S/T) PKA substrate antibody in control than in $(Bu)_2$-cAMP stimulated WT preparations. Fig. 6B shows that TPA and $(Bu)_2$-cAMP had similar increasing effects on the quantity of StAR; however, the intensity of the protein bands revealed by the anti-phospho-(S/T) PKA substrate antibody was much weaker for TPA than for $(Bu)_2$-cAMP preparations. Mutations S194A lowered the intensity of phospho-(S/T) bands in both situations. We thus took advantage of this situation to compare the effects of mutations S55A, S56A and S194A on StAR revealed by the anti-StAR antibody and by the anti-phospho-(S/T) PKA substrate antibody. We have made these comparisons on the preparations of COS-1 cells incubated with $(Bu)_2$-cAMP. With the anti-phospho-(S/T) PKA substrate antibody, the intensity of protein bands decreased by 56, 55, and 48% with mutations S55A, S56A and S194A, respectively. The quantity of StAR detected by the anti-StAR antibody differed very little between preparations, indicating that decreases in intensity, using the anti-phospho-(S/T) PKA substrate antibody, were due to the mutations and not to a decrease in the quantity of StAR per se (results not shown).

To completely understand the interrelation between the three phosphorylation sites revealed by the anti-phospho-(S/T) PKA substrate antibodies, we have engineered mutants containing the mutation S56A combined with other mutations at sites S55 or S194. Decreases in StAR phosphorylation of 44, 82, 99, 99 and 100% were found for mutants S194A, S55A/S56A, S56A/S194A, S55A/S194A and S55A/S56A/S194A for non-stimulated preparations and of 69, 78, 100, 94 and 100% for $(Bu)_2$-cAMP stimulated preparations, respectively (Fig. 7A). Under these conditions, StAR activity of the mutant S194A was decreased by 73 and 72%, respectively in control and $(Bu)_2$-cAMP stimulated preparations, whereas the activity of the triple mutant was decreased by 77 and 83%, respectively (Fig. 7B). This indicates that in this series of experiments the contribution of phosphorylation at sites S55 and/or S56 is much less important for StAR activity than that of site S194. Furthermore the remaining StAR activity (∼20%) thus appears to be independent of phosphorylation sites recognized by the anti-phospho-(S/T) PKA substrate antibodies.

4. Discussion

4.1. Pregnenolone production by COS-1 cells transfected with different StAR plasmids

A strong homology exists between StAR amino acid sequences of the 13 vertebrate species indexed in Table 1. We took advantage of the fact that many phosphorylation consensus sites were conserved among most of these species to elaborate a strategy to study putative StAR phosphorylation sites on the hamster StAR. In order to verify StAR activity alterations, we have used a previously validated StAR activity test model [36]. This test consists of the measure of cholesterol conversion into pregnenolone by COS-1 cells co-transfected with the expression plasmids of F2 and pcDNA3.1-StAR. We have established (Fig. 1) that a StAR/F2 plasmid ratio in the range of 0–75 ng/500 ng was suitable for the assay with or without $(Bu)_2$-cAMP

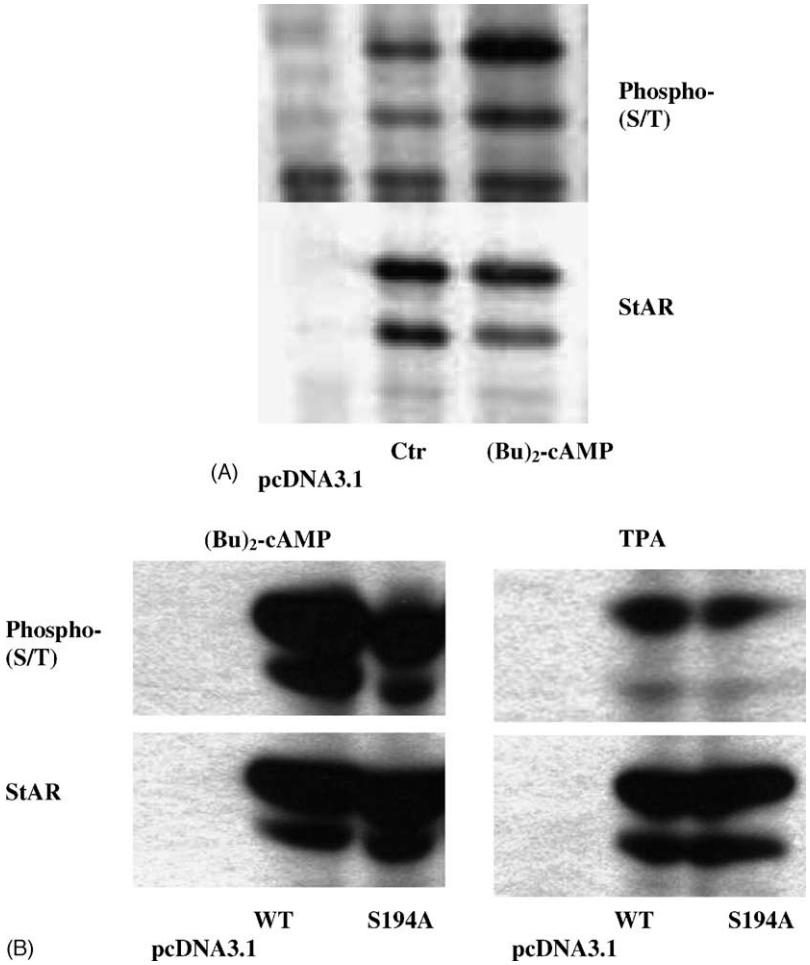

Fig. 6. Effects of $(Bu)_2$-cAMP and TPA on StAR phosphorylation. (A) COS-1 cells were co-transfected with the F2 construct (500 ng) and 500 ng WT-StAR-pcDNA3.1 plasmid for Ctr, 200 ng WT-StAR-pcDNA3.1 and 300 ng pcDNA3.1 plasmids for $(Bu)_2$-cAMP stimulated preparations, and 500 ng pcDNA3.1 plasmid for negative control; cells were incubated for 24 h. (B) COS-1 cells co-transfected with the F2 construct (500 ng), different StAR-pcDNA3.1 plasmids (200 ng) and pcDNA3.1 (300 ng), were incubated with $(Bu)_2$-cAMP (1 μm) or with TPA (32 nm) for 24 h. Immunoblotting was done on mitochondrial preparations using an anti-phospho-(S/T) PKA substrate antibody; after stripping, the same membranes were probed with an anti-StAR antibody.

stimulation. Indeed, at higher concentrations of StAR-pcDNA3.1 for transfections, the quantity of StAR continues to increase, especially under $(Bu)_2$-cAMP, while the production of pregnenolone reaches a plateau. Furthermore, differences in StAR activity that we observed between Ctr-WT, $(Bu)_2$-cAMP-WT and TPA-WT preparations was not due to a difference in the level of P450scc-adrenodoxin reductase-adrenodoxin expression. The expression levels of this protein complex, as analyzed by western blotting using either an anti-P450scc antibody or an anti-adrenodoxin antibody, were found similar under control and stimulated conditions (results not shown); therefore showing that the cholesterol side-chain cleavage system was not causing the changes in StAR activity under $(Bu)_2$-cAMP stimulation.

The basal activity of cells co-transfected with the empty pcDNA3.1 vector and F2 plasmid was not increased by the presence of $(Bu)_2$-cAMP indicating that a certain quantity of cholesterol was transformed into pregnenolone in the absence of StAR. This also indicates that $(Bu)_2$-cAMP alone did not act on the passive entry of cholesterol into mitochondria. Bose et al. [5] have also suggested StAR independent entry of cholesterol into mitochondria.

4.2. Mutants

In this study, the three most important changes in basal StAR activity were induced by mutations of S194, S185, and S55. However, the inhibition levels were noticeably different between these mutants. StAR activity was affected mostly by the mutations S194A and S185A and to a lesser degree by the mutation S55A. Dibutyryl cAMP was able to enhance the activity of S185A well above the WT basal activity whereas the stimulated activity of the S194A mutant remained below the WT basal activity, showing different mechanisms for these two mutants. The other putative phosphorylation sites tested involving S60, S68, S90, T262, S270 and S276 are not likely involved in StAR activity since removal of these putative phosphorylation sites did not affect pregnenolone formation nor the level of StAR expression (results not shown). The implication of S56 is discussed later.

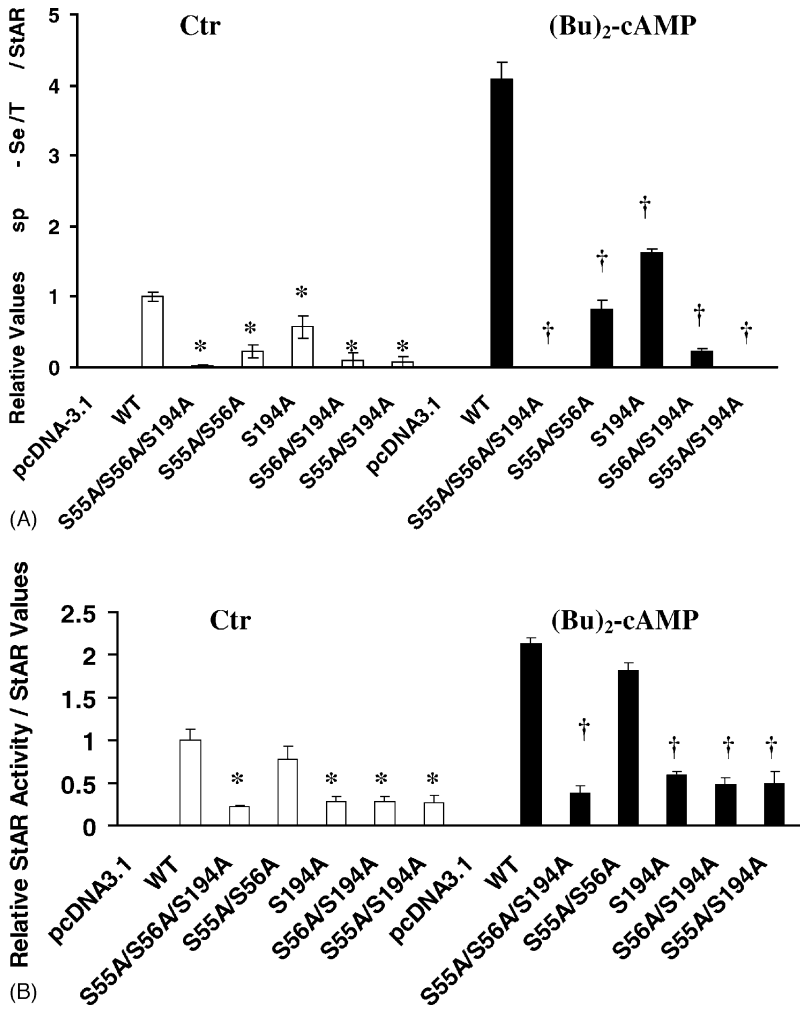

Fig. 7. Effects of $(Bu)_2$-cAMP on different StAR mutants. COS-1 cells were co-transfected with the F2 construct (500 ng) and different StAR-pcDNA3.1 plasmids (500 ng for control (Ctr); 200 ng for $(Bu)_2$-cAMP (1 mM)) and pcDNA3.1 (300 ng for $(Bu)_2$-cAMP) or 500 ng for negative control); cells were incubated for 24 h. Mitochondrial proteins from each preparation were separated by electrophoresis. Proteins were then transferred on membranes and revealed first by an anti-phospho-(S/T) PKA substrate antibody; after stripping, the same membranes were probed with an anti-StAR antibody. (A) Quantification of immunoblotting analyses expressed as phospho-(S/T) values/StAR values. (B) Relative StAR activity compared to non-stimulated WT-StAR levels. Values are the mean ± S.E.M. of three different experiments. $^*P < 0.05$ compared to Ctr-WT; $^\dagger P < 0.05$ compared to $(Bu)_2$-cAMP-WT.

We have demonstrated that the decreased activity of the mutant S185A was not due to a blockade of phosphorylation at this position. Substituting the amino acid S185 by either glutamic acid or aspartic acid in order to mimic the presence of a negatively charged group on this amino acid residue resulted in no increase but rather a decrease in StAR activity. Moreover, substitution of S185 by a cysteine residue to replace the serine OH group by a SH group having similar characteristics nearly fully restored basal and $(Bu)_2$-cAMP stimulated activities (Fig. 4A). In this series of experiments 200 ng of different StAR-pcDNA3.1 plasmids were used. At such a concentration of DNA, StAR activity is underestimated for samples stimulated by $(Bu)_2$-cAMP, which is not the case for controls (see Fig. 1); therefore comparisons can be made only within unstimulated or within stimulated preparations, respectively. Taken together these results thus clearly showed that S185 is not a phosphorylation site.

In contrast, the mutation S194C had no effect on basal StAR activity whereas the mutation S194D did enhance StAR activity (Fig. 5), indicating that S194 could in fact be a phosphorylation site. Furthermore, kinetic analyses (Table 2) confirm that the substitution of S194 by D, a negatively charged amino acid residue, confers similar kinetic properties to the mutant StAR to those of the WT $(Bu)_2$-cAMP stimulated preparations. These results favour the hypothesis that phosphorylation of S194 indeed increases StAR activity. This is an important observation since Tsujishita and Hurley [12] suggested that phosphorylation of this corresponding serine in MLN64 was not likely to affect the protein activity.

If S185 is not a phosphorylation site for the hamster StAR, then how can we explain that mutating this serine inhibited StAR activity? One explanation could be that mutations of S185 created StAR conformational changes resulting in a decrease in its binding affinity for cholesterol. This is reinforced

by the observation that although the $(Bu)_2$-cAMP stimulated WT and S185A mutant were similarly expressed and similarly processed by the mitochondrial machinery, the mutant activity remained much lower than the WT. Moreover the activity of the WT was comparable to the mild mutation S185C suggesting that only a small conformational change had occurred in the latter case. The conformational change produced by the mutations S185D and S185E however, should have been more drastic since they resulted in the complete abolition of basal as well as $(Bu)_2$-cAMP stimulated activity. This is mostly likely due to the drastic effect of altering a polar amino acid residue into a negatively charged amino acid residue.

To fully comprehend the effect of the mutations on the hamster StAR, we have constructed a molecular model for the hamster StAR. This model was elaborated according to crystallographic data of the human protein MLN64 [12]. MLN64 possesses a StAR related lipid transfer (START) domain found in StAR and in other proteins [41].

The details of the hamster StAR modelling are given in an article by Mathieu et al. [42]. Coordinates of the hamster StAR model are available at http://www.rcsb.org, PDB ID number 1ILJ. Briefly, the generated StAR model was very similar to the MLN64 model issued from crystallographic data. Like MLN64, the StAR model shows nine antiparallel β-sheet and four α-helices. Unlike the MLN64 crystallographic data, our model has a hydrophobic cavity instead of a hydrophobic tunnel. This tunnel, in MLN64, was proposed to be the site of cholesterol binding in MLN64 [12]. According to our model, the hamster StAR serine 194 is located to the exterior of the molecule and thus accessible to kinases. As mentioned earlier the hamster StAR amino acid residue S194 corresponds to the conserved S195 in human StAR that Arakane et al. [24] established to be phosphorylated upon $(Bu)_2$-cAMP stimulation. In contrast, S185 is hidden inside the protein and is consequently not accessible to kinases. However, S185 is located near the saline bridge formed by amino acid residues E168 and R187. As discussed by Mathieu et al. [42], strong evidence shows that this salt bridge is involved in the mechanism of cholesterol binding to StAR and translocation into mitochondria since it is the only charged component of the cholesterol-binding site, positioned properly for interaction with cholesterol through one molecule of water. This suggests that mutating S185, especially in the case of S185D and S185E, could have resulted in the perturbation of the saline bridge environment, explaining the inhibition of StAR activity.

4.3. Immunoblotting using an anti-phospho-(S/T) PKA substrate antibody

Under non-stimulated conditions, it was necessary to increase the quantity of StAR plasmid to 0.5 μg in cell transfections, in order to properly reveal differences in phosphorylation between the various mutant preparations studied. Under basal conditions the intensity of phosphorylated StAR revealed by the anti-phospho-(S/T) PKA substrate antibody was also decreased for S194A, S55A, S55A/S194A as well as for the S56A preparations. StAR activity was decreased for all preparations except for the S56A mutant.

Although the phospho-(S/T) intensity of the double mutant S55A/194A was additionally decreased compared to that of S194A or S55A single mutants, its activity was similar to that of the single mutants. We have thus speculated that when S55 was substituted by an alanine, maybe S56 could be phosphorylated instead to confer activity to the protein. To study this we have evaluated the effect of the triple mutation S55A/S56A/S194A on StAR phosphorylation and StAR activity.

Compared to WT, the triple mutation completely abolished detection by the anti-phospho-(S/T) PKA substrate antibody; however, contrary to our prediction, about 20% of StAR activity still remained under such conditions (Fig. 7B). We have reasoned that this remaining activity could be attributed to an underestimated StAR activity in WT preparations due the large quantity of StAR used for transfection. To answer this, a subsequent series of experiments using 75 ng of StAR-S55A/S56A/S194A, showed that the remaining activity in whole COS-1 cells was further decreased (to 10%) but not abolished in the mutant compared to WT preparations (results not shown). We thus speculate that the remaining StAR activity of the mutant S55A/S56A/S194A is due to a mechanism bypassing the normal StAR activation process.

An interesting fact is that at equal quantities of StAR, the anti-phospho-(S/T) PKA substrate antibody revealed four times more phosphorylation under $(Bu)_2$-cAMP stimulated than in control preparations (Fig. 6A). Under such conditions, StAR was twice less active in control than in stimulated preparations (Fig. 7B) emphasizing the correlation between phosphorylation and StAR activity. We believe that under basal conditions, a basal quantity of expressed StAR is possibly phosphorylated by the presence of endogenously activated PKA in COS-1 cells. The addition of $(Bu)_2$-cAMP into the incubation media results in more phosphorylated StAR available for cholesterol transfer into mitochondria.

In this study we have used different quantities of StAR-pcDNA3.1 for transfections. To establish a good correlation between the quantity of StAR and StAR activity under control and $(Bu)_2$-cAMP conditions, we have used quantities of DNA below 50–75 ng per 10×10^3 COS-1 cells. Under $(Bu)_2$-cAMP stimulation, with larger quantities of StAR-pcDNA3.1, the mitochondrial machinery necessary to transform cholesterol to pregnenolone (F2 plasmid) seemed to be saturated (Fig. 1). Indeed, with higher quantity of StAR-pcDNA3.1, the synthesis of StAR was augmented but this did not further enhance the synthesis of pregnenolone. To better detect changes in StAR under control conditions however, we had to increase the quantity of StAR-pcDNA3.1. Finally, to improve the detection of phosphorylated StAR species by the anti-phospho-(S/T) antibody, especially for control mitochondrial preparations, we had to use even larger quantities of StAR-pcDNA3.1.

The correlation between StAR phosphorylation and StAR activity is reinforced by the fact that TPA increased the quantity of StAR to a level comparable to that stimulated by $(Bu)_2$-cAMP but without increasing StAR activity (results not shown) nor StAR phosphorylation as analyzed with the anti-phospho-(S/T) PKA substrate antibody (Fig. 6B). This indicates that under the experimental conditions used, the protein kinase C pathway was not involved in controlling StAR activity in COS-1 cells. It is also possible that PKC isoforms necessary for StAR phosphorylation may not be expressed in COS-1 cells. Indeed in bovine adrenal *zona glomerulosa* cells, the PKC signalling pathway was effective in inducing StAR phosphorylation [43]. Similarly, we have found an enhanced StAR expression in vivo in the adrenal zona glomerulosa of rats fed a low sodium diet [16] which increases angiotensin II [13]. TPA was also shown to increase the expression of StAR in the human adrenal NCI-H295 cell line [44]. More work will thus be necessary to clarify the true role played by PKC, if any, in controlling StAR activity.

On another note, it was recently reported that the peripheral-type benzodiazepine receptor (PBR) and StAR come close enough to interact at the mitochondrial membrane surface [28]. Moreover, Papadopoulos et al. [45] reported a decrease in steroidogenesis in the absence of PBR. These data suggest that StAR and PBR could interact to promote cholesterol transfer to the mitochondrial internal membrane and are supportive that StAR can act on the outside of the outer mitochondrial membrane [46].

Our results are consistent with the model that upon translation, StAR becomes phosphorylated and interacts with mitochondrial components to favour cholesterol transport into the inner mitochondrial membrane. Phosphorylation of S194, S55 and perhaps S56 control StAR activity for the full length protein. We do not know yet if phosphorylation of these sites occurs before and/or after the interaction of StAR with mitochondrial components. However, the fact that non phosphorylated StAR species were found in isolated mitochondrial preparations (results not shown) is indicative that StAR can interact with this organelle without being phosphorylated.

5. Conclusion

To conclude, we have found that serines 55, 56 and 194 are three phosphorylation sites in the hamster StAR, which is in agreement with a recent mass spectrometry analysis demonstrating that StAR phosphorylated by protein kinase A led to the identification of serine 55/56 and serine 194 in rat StAR [47]. Compared to control at equal quantities, stimulation by $(Bu)_2$-cAMP results in a StAR that is more phosphorylated and more active. We have also found that mutations of amino acid serine 185, replacing it by aspartic acid or glutamic acid residues, are most likely to produce perturbations at the critically important cholesterol binding site salt bridge formed by amino acid residue E168 and R187. Consequently, this should alter the cholesterol binding site environment inhibiting StAR activity. Since a residual StAR activity was still observed for the S55A/S56A/S194A mutant, we speculate that this remaining activity is independent of phosphorylation at PKA sites, and could be due to the intrinsic activity of non phosphorylated StAR or to an artefact due to the large quantity of StAR expressed in COS-1. Finally, we have shown by kinetics studies that effectively $(Bu)_2$-cAMP provokes an augmentation of both the quantity and activity of StAR, and that an enhancement in StAR phosphorylation increases this activity. This contradicts the suggestion made by Tsujishita and Hurley [12] that serine 195 in the human StAR (S 194 in hamster) is not likely to be involved in StAR activity.

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