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Long-chain acyl-CoA synthetase 4 is regulated by phosphorylation

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ABSTRACT

Long chain acyl CoA synthetase 4 (Acsl4) is a key enzyme in steroidogenesis. It participates in steroid synthesis through of arachidonic acid release and Steroidogenic Acute Regulatory protein (StAR) induction.

Acsl4 prefers arachidonic acid as substrate and acts probably as a homodimer. In steroidogenic cells, it has been demonstrated that Acsl4 is a high turnover protein located mainly in mitochondrial-associated membrane fraction (MAM) bound to other proteins and that it is newly synthesized by hormone stimulation. The synthesis of Acsl4 constitutes an early step in steroidogenesis.

In the steroid synthesis process, activation of kinases plays a very important role. For this reason, the aim of this work was to study Acsl4 as a possible phosphoprotein and try to elucidate the role of its phosphorylation.

We have determined for the first time that Acsl4 is a phosphoprotein whose phosphorylation is hormone-dependent. We also demonstrated that Acsl4 acts effectively as a dimer and that phosphorylation occurs after dimer formation.

Studies *in vitro* demonstrated that Acsl4 is a substrate of both PKA and PKC and its phosphorylation by these kinases regulates its activity.

1. Introduction

Free fatty acids must be activated to their CoA thioesters before participating in most catabolic and anabolic reactions. Several processes such as incorporation of fatty acids into phospholipids or triacylglycerols, fatty acid elongation, unsaturation and degradation and fatty acylation of proteins require activated fatty acid substrates [1]. In turn, acyl-CoAs up-regulate uncoupling protein in brown adipose tissue and key enzymes of glycolysis, gluconeogenesis, and β-oxidation, are essential for vesicle trafficking, and play a critical role in the transport of fatty acids into cells by making transport unidirectional.

Free fatty acid activation is catalyzed by acyl-CoA synthetases, also known as acid:CoA ligases (AMP-forming) (EC 6.3.1.3). Acyl-CoA synthetases can be divided into five sub-families (1, 3, 4, 5 and 6) on the basis of the chain length of their preferred acyl groups. Long-chain acyl-CoA synthetases (Acsl) is the subfamily preferring fatty acids C12 to C20 as a substrate [2], that might act as homodimer [3].

In particular, Acsl4 shares 68% of its amino acid sequence with Acsl3 while it is poorly related to the other family members [4]. Purified Acsl4 prefers arachidonate as a substrate among other C8–C22 saturated and C4–C22 unsaturated fatty acids [2].

Tissue distribution is different for each Acsl. A striking feature of Acsl4 is its abundance in steroidogenic tissues, especially adrenal gland and ovary. Acsl4 immunoreactivity was been detected in the zona fasciculata and reticularis of the adrenal cortex, in the corpus luteum and stromal luteinized cells of the ovary and in Leydig cells of the testis [4]. Furthermore, Acsl4 is a peripheral membrane protein, located mainly on the mitochondrial-associated membrane fraction (MAM), on peroxisomal membrane and microsomes [5].

It has been demonstrated that Acsl4 expression in the Y1 murine adrenocortical tumor cell line is induced by ACTH and suppressed by glucocorticoids [6]. In Y1 and Leydig cells, its expression is also regulated by EGF, another factor that increases steroid production [7]. Furthermore, hormone stimulation of steroid production through the cAMP-dependent phosphorylation involves new synthesis of Acsl4 as an early step [8].

In hormone-induced steroidogenic cells Acsl4 activity appears to be essential for arachidonic acid release, StAR induction and steroid synthesis [8,9]. In Y1 and MA-10 cells, Acsl4 has been also proven as a high turnover protein [8].

Even if Acsl4 participation in steroidogenesis has been very well determined, its postransductional modifications have not been studied yet.

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Therefore, considering that Acsl4 is a high turnover protein which might act as a dimer, that it is induced by hormonal treatment and is located in MAM bound to other protein, events which could be regulated by phosphorylation, the aim of this work was to study whether Acsl4 is indeed a phosphoprotein and whether at least some of those events are actually regulated by protein phosphorylation.

The studies presented herein show that Acsl4 is a phosphoprotein whose phosphorylation is hormone-dependent and that this modification might regulate its activity.

2. Materials and methods

8Br-cAMP, 22(R)-OH-cholesterol, cycloheximide (CHX), PKC, arachidonic acid and Co-enzyme A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-39 kDa subunit of the NADH-cytochorme c reductase antibody was obtained from Molecular Probes, Inc. (Eugene, OR, USA). PKA catalytic subunit was from New England Biolabs (Beverly, MA, USA). All other reagents were of the highest grade available.

2.1. Cell culture

Murine Y1 adrenocortical tumor cells (ATCC CCL 79) were handled as described in [8].

MA-10 Leydig cell line was generously provided by Dr. Mario Ascoli, University of Iowa, College of Medicine (Iowa City, IA, USA) and was handled as originally described [10].

2.2. Preparation of mitochondrial fraction

Mitochondria and post-mitochondrial fractions (PMTC) were obtained as previously described [11].

2.3. Incorporation of [^{32}P]phosphate and Acsl4 immunoprecipitation

Y1 cultured cells were washed 3 times in phosphate-free Eagle media modified by Dulbecco (Gibco) and incubated for 5 h at 37 °C under 5% $C0_2$/95% air in the same medium containing [^{32}P]phosphate (200 μCi/ml). Then, EGF (10 ng/mL), ACTH (5 mU/mL) or 8Br-cAMP (1 mM) was added and incubated for another 10 min. The cells were washed three times with the media and lysed. Four hundred micrograms of cellular extract was used for Acsl4 immunoprecipitation as described in [8].

2.4. Assay of Acsl4 phosphorylation in vitro by PKA and PKC

Phosphorylation assay by PKA was performed in a final volume of 30 μL containing 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM EGTA, 1 mM, β-mercaptoethanol, 0.1 mM [γ-^{32}P] ATP 10 μCi, 10 UI of PKA catalytic subunit and 3 μg of purified Acsl4 at 30 °C.

Phosphorylation assay by PKC was performed in a final volume of 30 μL containing 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 5 μg phosphatidylserine, 0,4 μg diacylglycerol, 10 μM [γ-^{32}P]ATP 10 μCi, 10 UI PKC catalytic subunit and 3 μg of purified Acsl4 at 30 °C. In the indicated cases kinase reaction was started without radioactive ATP, which was later incorporated. After the phosphorylation assays, proteins were separated by SDS–PAGE, transferred to PVDF membrane and exposed to X-ray film to detect phosphorylated proteins. Then, a Western Blot for Acsl4 detection was made.

For quantification of phosphate incorporated per mole of protein, gels were dried, phosphorylated bands were cut and radioactivity was measured by liquid scintillation counting.

When sequential phosphorylations were done, the reaction conditions were those used for PKC reaction but using 0.1 mM ATP instead.

2.5. Acsl4 activity reaction

Acsl4 was previously phosphorylated by PKA or PKC for 30 min as stated above. Then Acsl4 activity reaction was carried out in a final volume of 90 μL for 30 min at 37 °C in the following conditions: 100 mM Tris–HCl (pH 7.4), 5 mM ATP, 250 μM Coenzyme A, 50 μM arachidonic acid, 0.03% Tritón X-100, 1 μM EDTA, 8 mM $MgCl_2$, 5 mM DTT, 0.4 μCi [$^{1-14}$C] arachidonic acid. The reaction was stopped by the addition of 1% ClH (0.8 mL) and then 4 extractions with cold n-hexane were made. The aqueous phase was measured by liquid scintillation counting.

2.6. Western Blot analysis

Proteins were separated by SDS/PAGE and transferred to poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) as described previously [9]. For separation in non-reducing condition, β-mercaptoethanol free sample buffer was used.

In the case of bidimentional electrophoresis, first dimension isoelectric focusing was done using Immobiline DryStrip precast gels (Amersham) (pH 6–11, 7 cm) according to manufacturer's recommendations. The second dimension was done as indicated above.

Acsl4 protein was detected using anti-Acsl4 antibodies [8] and enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, UK). For quantitative analysis, band intensities were analyzed using ImageQuant 5.2 software.

2.7. Plasmid transfection

MA-10 cells were transiently transfected with pcDNA3 plasmid containing Acsl4 cDNA or empty plasmid as indicated in [7].

2.8. Protein determination and statistical analysis

Protein was quantified by Bradford's method [12] using bovine serum albumin as standard. Statistical analysis was performed by ANOVA followed by the Student–Newman–Kuels test.

3. Results

3.1. Is Acsl4 a phosphoprotein?

Given that steroidogenic hormones trigger kinase activation and that some events as subcellular localization, dimer formation and protein half-life could be regulated by addition of phosphate group, we studied whether Acsl4 can be phosphorylated by hormonal stimuli. For this purpose we immunoprecipited Acsl4 of Y1 cells that were incubated with [^{32}P]phosphate and stimulated by either ACTH, its second messenger 8Br-cAMP or EGF, another well known factor that induces steroidogenesis [7] (Fig 1A).

As it is shown in the autoradiography, we observed a radioactive band of 74 kDa. The identity of this band was confirmed by Western-Blot analysis for Acsl4, which indicated that it is indeed a phosphoprotein (Fig 1B). On the other hand, radioactivity was higher in Acsl4 from stimulated cells compared to the control ones (Fig 1C). This result would indicate that hormonal stimuli induce Acsl4 phosphorylation.

In order to determine Acsl4 phosphorylated forms, proteins from control and stimulated cells were separated by bidimensional

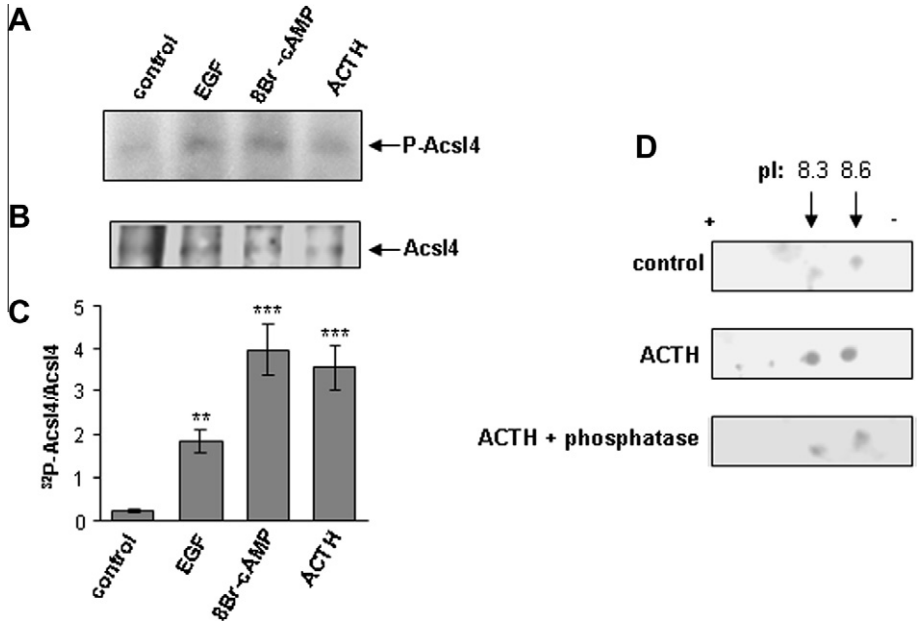

Fig. 1. Acsl4 is a phosphoprotein. Y1 cells were labeled for 5 h with [^{32}P]phosphate (1 mCi/ml) and incubated in the presence of EGF (10 ng/mL), ACTH (5 mU/mL) or 8Br-cAMP (1 mM) for 10 min. Then, Acsl4 was immunoprecipitated, subjected to SDS–PAGE and transferred to a PVDF membrane. Panel (A): A representative autoradiography of the membrane, Panel (B)+: Western Blot for Acsl4, Panel (C): quantification of band intensity of three independent experiments; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control and Panel (D): Y1 cells were incubated in the presence or in the absence of 5 mU/mL ACTH and the proteins obtained were incubated with acid phosphatase or its vehicle before 2D-electrophoresis separation and Western Blot for Acsl4. Western Blot representative of three independent experiments is shown.

electrophoresis and analyzed by Western-Blot using anti-Acsl4 antibody. As shown in Fig 1D, two spots of Acsl4 were present in control cells. When the cells were hormone-stimulated, two new spots at more acidic pH were found, which suggests phosphoprotein forms. In order to confirm whether these spots are indeed phosphorylated forms, proteins from stimulated cells were incubated with phosphatase before bidimensional electrophoresis separation. As it is shown in Fig 1D, the two most acidic spots were absent when proteins were before incubated with phosphatase, which confirmed the presence of phosphate groups in these spots.

3.2. Acsl4 is a substrate of PKA and PKC in vitro

Since PKA or PKC activation (depending on cellular type) is a necessary event for steroid production, we first analyzed the presence of probable phosphorylation sites by in silico analysis of the aminoacidic sequence of Acsl4 using the NetPhosK 1.0 software. This analysis determined that Acsl4 contained probable phosphorylation sites for these kinases with a high score. It is worth pointing out that one site (Ser 318) is shared by both PKA and PKC.

Then we analyzed whether Acsl4 is a substrate of these kinases *in vitro*. To this end, we purified Acsl4 as a chimeric protein with Glutation S-transferase (101.5 kDa) from Escherichia coli, as indicated in [8], and used it in phosphorylation assays using PKA or PKC as kinase. We determined that Acsl4 is phosphorylated by both kinases in a time-dependent form as it is shown in Fig 2A and B. As indicated, PKC is autophosphorylated in this assay.

The quantification of phosphate incorporated per mole of protein was 0.55 and 0.62 mol by PKA and PKC respectively measured after 150 min of incubation. At this time the product concentration in both reactions reached its maximum (data not shown). These results indicated that Acsl4 is phosphorylated only in one site for each kinase.

In order to determine the possible connection between PKA and PKC phosphorylations, purified Acsl4 was phosphorylated according to the scheme indicated in Fig 2C.

Equivalent phosphorylation was obtained when the reaction was carried out with the combination of both kinases, regardless of whether PKA or PKC was used first (Fig 2, treatment 1 and 4). On the other hand, it should be noted that, despite 30 min of non-radioactive kinase reaction, phosphorylation continued during the following 30 min, as it is shown in treatments 3 and 6. Furthermore, the phosphorylation level reached during the sequential kinase reactions was equal to the addition of phosphorylation levels reached by each of the enzymes separately. This result allows us to conclude that PKA and PKC phosphorylations are independent from one another. Furthermore, this data indicates that these kinases phosphorylate Acsl4 at different sites.

Interestingly, Acsl4 seems to act as a homodimer, as suggested by crystal structure of long-chain fatty acyl-CoA synthetase from Thermus termophilus (ttLC-FACS) [3], the only well defined structure for this protein family. The dimer formation is regulated by phosphate incorporation in several proteins. As we demonstrated that Acsl4 obtained from *E. coli* was a substrate of PKA and PKC, it was necessary to analyze whether this protein appeared in a monomeric o dimeric form. To this end, we separated both forms by a size-exclusion chromatography and found out purified Acsl4 to have a dimeric form (data not shown). According to results, the phosphorylation event does not seem to be essential for Acsl4 dimer formation.

An unexpected result was observed in Acsl4 Western-Blot studies following electrophoresis in non-reducing conditions, where β-mercaptoethanol free sample buffer was used instead. In that condition, a band of 150 kDa was present in samples from both control and hormone-stimulated cells (Fig 3A), consistent with Acsl4 dimeric form. This band disappeared when the samples were subjected to reducing conditions and only the monomeric form (74 kDa) was evident, which indicates that the 150 KDa band was indeed a dimeric form of Acsl4. Consequently, we can conclude that Acsl4 has a dimeric form in mammalian cells, and that hormone-stimulation is not necessary for its formation.

In order to determine whether Acsl4 phosphorylation occurs in the monomeric o dimeric form, we immunoprecipitated Acsl4

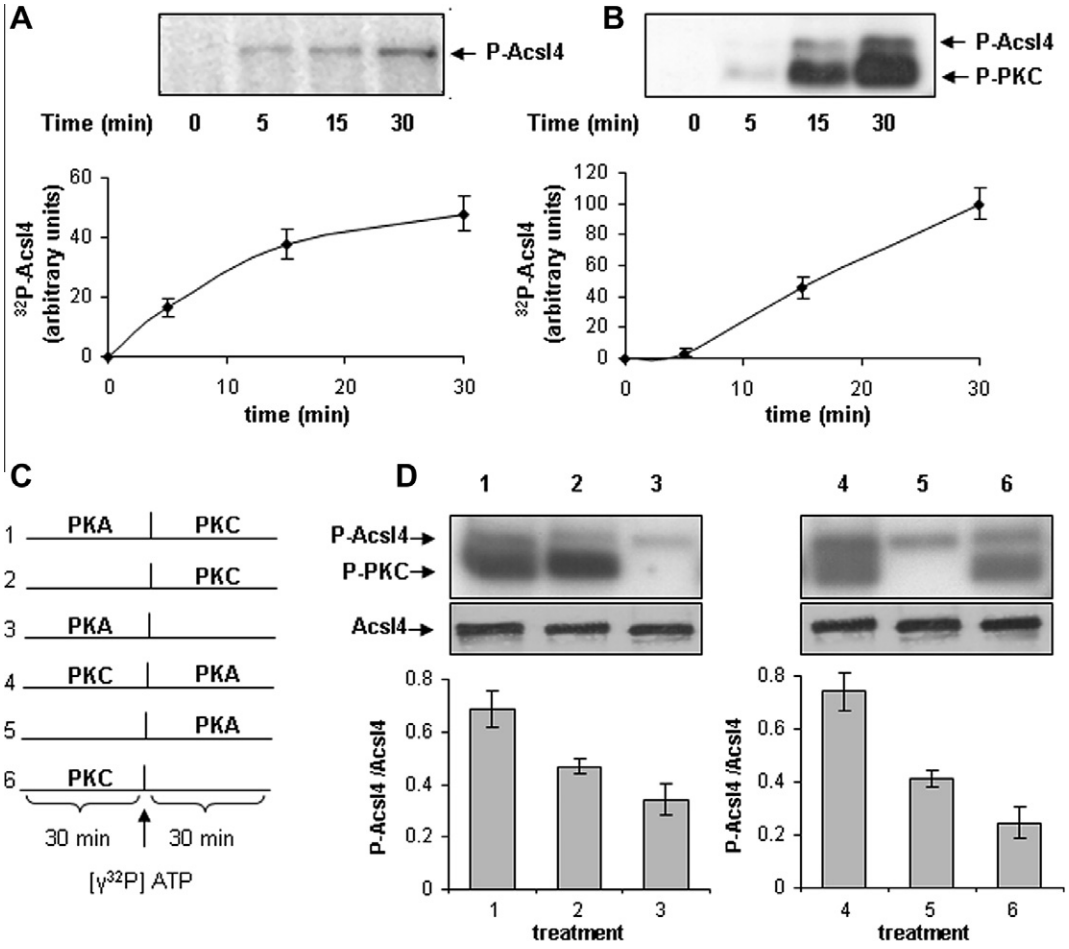

Fig. 2. Acsl4 is a PKA and PKC substrate *in vitro*. Acsl4 was phosphorylated by PKA (Panel A) or PKC (Panel B) for different times. In both cases, the autoradiography is shown in the upper panels. In the Panel B, the PKC autophosphorylation band appears under Acsl4 phosphorylated ones. The intensity of Acsl4 bands from three independent experiments was quantified and plots obtained are shown in lower panels, Panel (C): scheme used for sequential phosphorylation assays to study interdependency between PKA and PKC phosphorylation. The kinase reactions during the first 30 min were done in the absence of radioactive ATP and in the presence of the indicated kinase; then [γ^{32}P]ATP and the other kinase were added and the reaction was incubated for 30 min, and Panel (D): a representative autoradiography of the membrane and Western Blot for Acsl4 are shown in the upper panels. The PKC autophosphorylation band appears under Acsl4 phosphorylated ones. Band intensity of Acsl4 from three independent experiments was quantified and results are shown in the lowers panels. Data represent mean ± SD of three independent experiments.

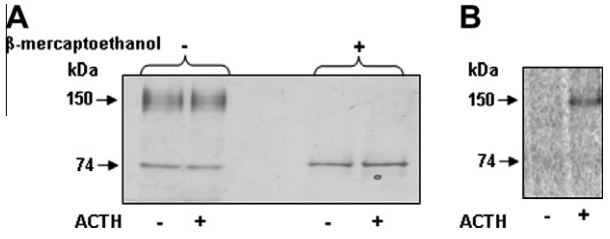

Fig. 3. Study of monomeric and dimeric forms of Acsl4. Panel (A): Y1 cells were incubated in the presence or in the absence of ACTH for 10 min and the proteins obtained were placed in sample buffer with or without β-mercaptoethanol before electrophoresis and Western Blot for Acsl4. Western Blot representative of three independent experiments is shown and Panel (B): Y1 cells were labeled for 5 h with [^{32}P]phosphate (1 mCi/ml) and incubated in the presence of ACTH (5 mU/mL) for 10 min. Then, Acsl4 was immunoprecipitated and subjected to SDS–PAGE in non-reducing condition. Gel autoradiography is shown.

from cells incubated in the presence of [^{32}P] phosphate and separated by non-reducing electrophoresis and analyzed phosphate incorporation. As autoradiography in Fig 3B shows, the phosphate was incorporated only in the dimeric form when cells were hormone-stimulated, which indicates that phosphorylation is not a necessary event for dimer formation and that it might have a different role.

3.3. Small advances in the study of the effects of Acsl4 phosphorylation

It is well known that Acsl4 expression is induced by hormonal treatment in sterodogenic cells [8], but the localization of the newly synthesized protein has not been determined. Therefore, we studied the effect of hormonal stimulation on Acsl4 subcellular localization. For that purpose, MA-10 Leydig cells were stimulated by 8Br-cAMP, the LH permeable second messenger, and the mitochondrial and post mitochondrial fractions (PMTC) were obtained (Fig 4A).

As it was expected, 8Br-cAMP stimulation increased Acsl4 expression. This newly synthesized protein localized in mitochondria. It should be noted that the MAM fraction was obtained in the mitochondrial preparation. On the other hand, in the post mitochondrial fraction, a decrease in Acsl4 level was observed.

In order to determine the effect of phosphorylation on Acsl4, we analyzed the subcellular localization of Acsl4 over-expressed in MA-10 cells. As Fig 4B shows, Acsl4 over-expressed is located in the mitochondrial fraction regardless of hormonal stimuli. This seems to suggest that phosphorylation does not participate in the determination of Acsl4 mitochondrial localization.

To evaluate whether phosphorylation modifies Acsl4 activity, we measured this activity *in vitro* using purified Acsl4 previously

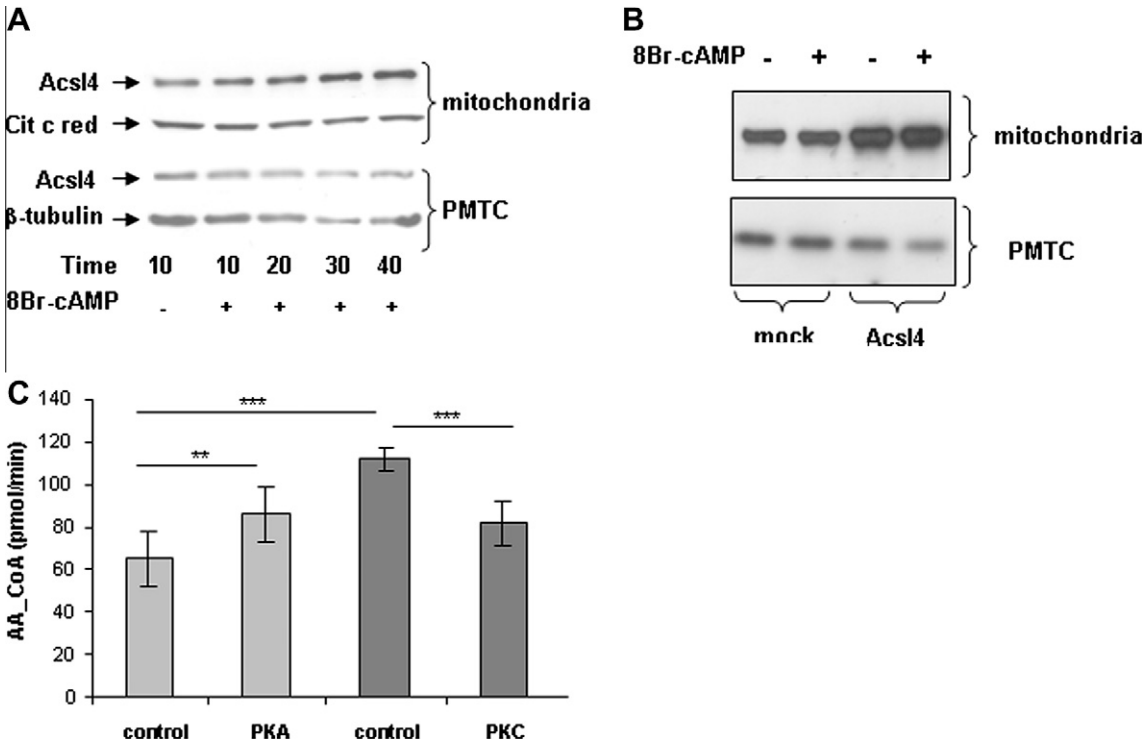

Fig. 4. Studies of Acsl4 phosphorylation effects. Panel (A): MA-10 cells were incubated in the presence of 1 mM 8Br-cAMP for the indicated times and mitochondrial and postmitochondrial (PMTC) fractions were obtained. The proteins were separated by SDS–PAGE and Acsl4 expression was evaluated by Western Blot. Mitochondrial 39 kDa subunit of NADH-cytochrome c reductase (cyt c red) and cytosolic β-tubulin were used as loading control. Western Blot representative of three independent experiments is shown, Panel (B): MA-10 cells were transiently transfected with a plasmid containing Acsl4 cDNA (pcDNA3-Acsl4) or empty plasmid (mock). Forty-eight hours after transfection, mitochondria and PMTC fractions were isolated and Acsl4 protein levels were evaluated by Western Blot and Panel (C): Acsl4 activity in its non-phosphorylated and PKA or PKC-phosphorylated forms was measured. Data represent the means ± SD of three independent experiments. $^{**}P$, 0.01 and $^{***}P$, 0.001.

phosphorylated by PKA or PKC. As Fig 4C shows, Acsl4 is active in its non-phosphorylated state. The control activity was higher when the reaction was made in the presence of phospholipids needed for PKC kinase assay. However, when Acsl4 was PKA-phosphorylated, its activity was significantly increased compared to the activity of non-phosphorylated Acsl4. On the contrary, PKC phosphorylation caused a significant reduction in the Acsl4 activity.

4. Discussion

The present study aimed at investigating whether Acsl4 is a phosphoprotein and some aspects of Acsl4 that could be regulated by phosphorylation in steroidogenic cells.

By phosphate incorporation and immunoprecipitation, we have demonstrated that Acsl4 is a phosphoprotein and that its phosphorylation is hormone-regulated. This was verified by bidimensional electrophoresis, as two new more acidic spots of Acsl4 were present when cells were hormone-stimulated. These spots were absent when proteins were incubated in the presence of phosphatase, which indicates that they were indeed phosphorylated forms. The presence of two spots from control cells and phosphatase-resistant spots could be due to the presence of Acsl4 isoforms by alternative splicing [2].

The only member of this family in which this postrasductional modification was previously determined was a poorly related member, ACSL1 [13], an integral membrane protein. To our knowledge, this is the first time that Acsl4 has been shown to be a phosphoprotein.

Acsl4 has been found in MAM, peroxysomes and microsomes in different kinds of cells. We have determined that Acsl4-newly synthetized by hormonal stimuli– localizes in MAM. Since steroid synthesis takes place in mitochondria and Acsl4 has a very important role in this event, it is logical to think that the increment might occur in this compartment. Also, localization does not seem to depend on protein phosphorylation, as over-expressed Acsl4 was also localized in mitochondria in control cells.

There is some evidence indicating that all long-chain fatty acyl-CoA synthetases could act as homodimers, as suggested by the ttLC-FACS crystal structure [3]. In this protein the homodimer is formed by multiple intermolecular salt bridge interactions. We show in this work that Acsl4 structure acts as a homodimer. Strikingly, this phenomenon was observed when electrophoresis was conducted in non-reducing conditions, which indicate that a disulfide bridge is necessary for Acsl4 dimer formation.

We also found out that purified rat Acsl4 from of *E. coli* transformed with plasmid containing rat Acsl4 sequence has a homodimeric form, which indicates that dimer formation can occur without previous phosphorylation. Furthermore, Acsl4 phosphorylation takes place once the dimer is established.

PKA and PKC play an important role in steroid synthesis and, in turn, Acsl4 is known to be essential for steroidogenesis. Therefore, we analyzed the possibility that this protein might be a substrate for these kinases. Noteworthy, the most probable phosphorylation sites were those corresponding to these kinases, as established by in silico studies. *In vitro* phosphorylation studies showed Acsl4 to be a substrate of both PKA and PKC. Phosphorylation for each of them is independent and occurs in a different site.

We analyzed Acsl4 activity in its phosphorylated and non-phosphorylated forms. Rat Acsl4 obtained from bacteria evidenced a basal activity that increased when lipids were present. Lipids have been shown to enhance the Acsl activities and the lipid composition of the membranes in which the enzymes are measured has been reported to affect their activity [14].

In *in vitro* studies phosphorylation produced changes in Acsl4 activity, increasing it when Acsl4 was PKA-phosphorylated, and reducing it when the protein was PKC-phosphorylated. The rapid Acsl4 activity regulation ensures that cells can respond to constantly changing conditions and that the protein is active only when necessary; otherwise, it could increase the amount of product, i.e. arachidonoyl-CoA, which can cause damage in the cell by its detergent effect. Even if further studies will be required to establish whether Acsl4 is an in vivo substrate of PKA and PKC, our results indicate it is highly probable.

In conclusion, to our understanding, this is the first time that Acsl4 has been proven as a phosphoprotein. Also, we indicate that phosphorylation might play a role in events necessary for steroid synthesis triggered by steroidogenic hormones.

More studies will be necessary to completely understand the functional consequences of this postransductional modification.

Since Acsl4 plays a very important role in other relevant events like tumor promotion in breast cancer cells and colon cancer cells [15] and in maturation and remodeling of neurons, among others, this postraductional modification has to be taken into account.

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