Protein tyrosine phosphatases are involved in LH/chorionic gonadotropin and 8Br-cAMP regulation of steroidogenesis and StAR protein levels in MA-10 Leydig cells

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Abstract

The LH signal transduction pathway features the activation of protein tyrosine phosphatases (PTPs) as one of the components of a cascade that includes other well characterized events such as cAMP-dependent protein kinase A (PKA) activation. Moreover, the action of PTPs is required to increase the rate-limiting step in steroid biosynthesis, namely the cAMP-regulated transfer of cholesterol to the inner mitochondrial membrane. Since both PKA activity and steroidogenic acute regulatory (StAR) protein induction are obligatory steps in this transfer of cholesterol, the present study was performed to investigate the role of PTPs in the regulation of PKA activity and StAR expression in response to LH/chorionic gonadotropin (CG) and 8Br-cAMP in MA-10 cells. While the exposure of MA-10 cells to the PTP inhibitor, phenylarsine oxide (PAO), did not modify PKA activity, it partially inhibited the effect of human CG and cAMP analog on StAR protein levels. Time-course studies demonstrated that PAO inhibited cAMP induction of StAR protein and mRNA. At 30 min, the effect on cAMP-stimulated StAR protein levels was a 35% inhibition, progressing to up to 90% inhibition at 120 min of stimulation. The maximal inhibitory effect on cAMP-induced StAR mRNA level was obtained at 60 min (85%). In summary, these results demonstrate that inhibition of PTP activity affected both StAR protein and mRNA synthesis and suggest that the activity of hormone-regulated PTPs is a requirement in the LH signaling cascade that results in the up-regulation of StAR protein and, subsequently, increased steroid synthesis.
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Introduction

Testicular steroid production is controlled by luteinizing hormone (LH), secreted from the anterior pituitary. While LH is the major physiological agonist in most animals, its action can also be mimicked by chorionic gonadotropin (CG). The signal transduction cascade involved in LH/CG action includes increases in intracellular cAMP levels and activation of protein kinase A (PKA), events that also occur in adrenocorticotropin (ACTH)-stimulated steroid production in adrenal cortical cells (Cooke *et al.* 1976, Dufau *et al.* 1977, Podestá *et al.* 1979, Rae *et al.* 1979, Sala *et al.* 1979, Wang & Ascoli 1990, Swinnen *et al.* 1991). It is well recognized that one of the requirements for full steroidogenic activity in response to LH/CG and ACTH is the PKA-mediated phosphorylation of regulatory proteins (Pon *et al.* 1986, Chaudary & Stocco 1991, Stocco & Clark 1993).

The rate-limiting and acutely regulated step in hormone-stimulated steroidogenesis in the adrenals and gonads is the delivery of cholesterol from the outer to the inner mitochondrial membrane where it is metabolized to pregnenolone by the cytochrome P450 cholesterol side chain cleavage enzyme (P450 scc) (Crivello & Jefcoate 1980, Privalle *et al.* 1983). Since this enzyme resides on the matrix side of the inner mitochondrial membrane, the assisted delivery of cholesterol to this site is critical for the rapid synthesis of steroids in response to stimulation. Numerous reports demonstrate that the steroidogenic acute regulatory (StAR) protein plays a critical role in the regulation of this step by mediating cholesterol transfer to the P450 scc enzyme (Clark *et al.* 1994, Stocco & Clark 1996). In this regard, perhaps the most striking evidence of the role of StAR in hormone-induced steroid synthesis is the demonstration that mutations in the human StAR gene cause the disease congenital lipoid adrenal hyperplasia, a potentially lethal condition in which adrenal and gonadal steroidogenesis is virtually eliminated (Lin *et al.* 1995). The further characterization of StAR has revealed that this protein may be considered as the 'labile protein'

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that mediates the steroidogenic response to hormone action in Leydig and adrenal cells, as well as in other steroidogenic systems studied to date. Thus, StAR is rapidly synthesized in response to the respective tropic hormones, its active form has a very short half-life, and it rapidly increases the transport of cholesterol to the site of P450 scc action (Stocco & Clark 1996).

Recent evidence has also indicated that both the LH/CG and the ACTH signal transduction pathways include, as an additional component, protein tyrosine dephosphorylation mediated by hormone-regulated protein tyrosine phosphatases (PTPs). As such, we have previously demonstrated in rat adrenal zona fasciculata (Paz *et al.* 1999), and in both primary Leydig cell cultures and MA-10 cells (Cornejo Maciel *et al.* 2001), that protein tyrosine phosphatase inhibitors notably reduced the induction of steroid biosynthesis in response to either tropic hormone or cAMP analog. In contrast, these inhibitors had no effect on the level of steroids synthesized from 22R-hydroxy cholesterol (22R HC) that was added to the medium (Paz *et al.* 1999, Cornejo Maciel *et al.* 2001). 22R HC is a cholesterol analog that freely diffuses to the P450 scc enzyme, thus obviating the need for StAR and indicating that the enzymes for steroid conversions are active. In addition, we have demonstrated that human (h) CG and 8Br-cAMP produce a rapid and transient increase in total PTP activity in MA-10 cells that involves at least two PTPs (110 kDa and 50 kDa forms), and induce tyrosine dephosphorylation of endogenous proteins (Cornejo Maciel *et al.* 2001). Moreover, we and others have described similar effects following ACTH treatment of bovine (Vilgrain *et al.* 1998) and rat (Paz *et al.* 1999) zona fasciculata cells.

The results obtained with 8Br-cAMP-stimulated and 22R HC-supported steroidogenesis indicate that PTP action is restricted to a point located between the PKA phosphorylation of putative regulatory proteins and the translocation of cholesterol into the mitochondria. Taking into account the well recognized roles played by the PKA-mediated protein phosphorylation and StAR protein in the rate-limiting step in steroidogenesis these observations led us to hypothesize that the target of PTP activity could be at the level of PKA activation or StAR protein synthesis. Therefore, the present study was undertaken to investigate the role of PTPs in the regulation of PKA activity and StAR expression in response to LH/CG and 8Br-cAMP in MA-10 cells.

Materials and Methods

Cell culture of MA-10 Leydig cells

The MA-10 cell line is a clonal strain of mouse Leydig tumor cells that produce progesterone rather than testosterone as the major steroid. The cells were generously provided by Dr Mario Ascoli, University of Iowa, College of Medicine (Iowa City, IA, USA) and were handled as originally described (Ascoli 1981). The cell line was maintained in 75-cm^2 flasks and experiments were performed in 6 × 35-mm wells in multi-well plates. The growth medium consisted of Waymouth MB752/1 (GIBCO-Life Technologies) containing 1·1 g/l $NaHCO_3$, 20 mM Hepes, 50 μg/ml gentamycin, and 15% horse serum. Flasks and multi-well plates were maintained at 36 °C in a humidified atmosphere containing 5% CO_2.

Conditions for incubation of MA-10 cells with PTP inhibitors

Treatment of MA-10 cells with hCG and 8Br-cAMP was performed in serum-free medium supplemented with 1 mg/ml BSA at final concentrations of 10 ng/ml or 1 mM, submaximal and maximal doses respectively. Purified human CG (hCG CR-127, potency 14 900 IU/mg) was a gift from Dr Parlow, National Hormone and Pituitary Program, NIDDK, NIH (Bethesda, MD, USA). Stimulations were performed in the presence or absence of phenylarsine oxide (PAO), a cell-permeable PTP inhibitor (García Morales *et al.* 1990, Wei *et al.* 2000), added to the cells 10 min before the stimuli. Following the appropriate treatments, MA-10 cells were placed in an incubator at 36 °C and 5% CO_2 for the time periods indicated in the Figure legends. Following incubations, media and cells were collected. Media were stored at −20 °C until progesterone was assayed by radioimmunoassay and the cells were retained for subsequent Western and Northern analysis.

Western blot and Northern blot analysis

Following the appropriate treatments, MA-10 cell cultures were washed with phosphate-buffered saline, scraped into a buffer containing 10 mM Tris, pH 7·4, 250 mM sucrose, 0·1 mM EDTA, homogenized in a glass–Teflon pestle homogenizer, and centrifuged at 600 ***g*** for 15 min. A second centrifugation at 10 000 ***g*** for 15 min resulted in a supernatant and a mitochondrial pellet.

StAR protein content in the mitochondrial protein pellets was assessed by Western analysis. Mitochondrial proteins (usually 25–50 μg per sample) were separated by SDS gel electrophoresis using 12% polyacrylamide gels as described by Laemmli (1970) and electrophoretically transferred to nitrocellulose membranes according to the procedure described by Towbin *et al.* (1979). StAR protein was detected using anti-StAR antibodies and immunoreactive bands were visualized by enhanced chemiluminescence as previously described (Clark *et al.* 1994).

For Northern analysis, total RNA was isolated from MA-10 cells by the guanidinium isothiocyanate method using TriZol reagent (GIBCO-Life Technologies), according to the manufacturer's protocol. Twenty-four

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micrograms total RNA were separated by electrophoresis on 1·5% agarose gels and blotted onto Hybond N+ (Amersham). StAR mRNA was detected using a specific ^{32}P-labeled cDNA probe.

For quantitative analysis, densitometry was performed on autoradiograms using an image analyzer (BioImage Visage 2000, Ann Arbor, MI, USA).

PKA determination

The effect of PAO on PKA activity was determined by measuring the incorporation of [^{32}P]orthophosphate from [^{32}P]γ-ATP into the PKA peptide substrate (Kemptide, New England BioLabs, Beverly, MA, USA). We tested both the activity of a recombinant PKA catalytic subunit (New England BioLabs) and the enzyme present in MA-10 cells incubated for 15 min or 1 h with or without 8Br-cAMP (1 mM) in the presence or absence of PAO (2 μM) or H89 (20 μM), a recognized PKA inhibitor. Following the treatment, MA-10 cell cultures were washed with phosphate-buffered saline, scraped into a buffer containing 25 mM Tris–HCl, pH 7·4, 0·5 mM EDTA, 0·5 mM EGTA, 1 μg/ml leupeptin and 1 μg/ml aprotinine, and sonicated by three 15 s cycles in a Branson 250 sonicator (Branson Ultrasonics Corp., Dansbury, CT, USA) at 70 W. This material was used for PKA activity determinations.

Kemptide (50 μM) was incubated with 4 μg of cellular proteins in a reaction mixture consisting of 50 mM Tris–HCl, pH 7·5, 10 mM $MgCl_2$, 200 μM ATP (100 c.p.m./pmol) (25 μl of total volume). The *in vitro* effect of PAO was assessed using ≅ 0·5 unit of enzyme (recombinant catalytic subunit) and performing the reaction in the presence or absence of 2 μM PAO. The reaction was allowed to proceed for 10 min at 30 °C and was terminated by spotting onto p81 paper (Whatman) and washing in 75 mM orthophosphoric acid, as previously described (Garton & Tonks 1994).

Determination of total cellular protein synthesis

In order to determine the effects of PAO on total protein synthesis, MA-10 cell cultures were incubated as described above with the inclusion of 20 μCi/ml ^{35}S-methionine (specific activity 1175 Ci/mmol; New England Nuclear) for 20 min. Following this pre-incubation period, 1 mM 8Br-cAMP was added either alone or in the presence of 2 μM PAO. After the periods of time indicated in Table 2, the media were removed, the cells were washed and then homogenized in a buffer consisting of 10 mM Tris, pH 7·4, 250 mM sucrose and 0·1 mM EDTA. Total incorporated radioactivity in an aliquot of the homogenate was quantitated. An equivalent aliquot was used to precipitate proteins using an equal volume of 20% trichloroacetic acid (TCA). Precipitated proteins were transferred onto glass fiber filters that were washed and dried before counting the radioactivity in a β counter. Results are expressed as the percentage of the total cellular radioactivity incorporated into proteins.

Protein determination

Protein concentration was determined by the method described by Lowry *et al.* (1951), using BSA as standard.

Statistical analysis

Each experiment was performed at least three times under identical conditions. Unless otherwise indicated, results were analyzed separately, and a representative experiment is shown in the figures. Values represent the mean ± S.D. Statistical significance was evaluated using ANOVA followed by Tukey's test. $P<0·05$ was considered significant.

Results

Determination of protein kinase A activity

It has previously been reported that PKA-mediated protein phosphorylation is necessary for the stimulation of steroidogenesis and StAR action in MA-10 cells (Swinnen *et al.* 1991, Stocco & Clark 1993, Arakane *et al.* 1997). In addition, we have already demonstrated that protein tyrosine phosphatases inhibition by compounds like PAO results in a reduced production of steroids under hormone and cAMP stimulation (Paz *et al.* 1999, Cornejo Maciel *et al.* 2001). One possibility is that the action of protein tyrosine phosphatases on steroid production is exerted on PKA activation and/or activity itself. To test this possibility, we studied the effect of PAO on the activity of a recombinant PKA catalytic subunit and on the activity of endogenous PKA of MA-10 cells exposed to PAO.

Previous studies indicated that the exposure of MA-10 cells to 1 μM PAO for a time as short as 10 min was enough to inhibit PTP activity by 50% (Cornejo Maciel *et al.* 2001). In addition, this compound caused a concentration-dependent inhibition of hCG- and 8Br-cAMP-induced progesterone production in these cells over the range of 0·5–2 μM. The highest concentration produced a 90–95% inhibition of stimulated steroid production and it did not affect basal or 22R HC-supported progesterone production (Cornejo Maciel *et al.* 2001). Based on these previous results, we evaluated the effects of 2 μM PAO on both PKA activity and StAR protein levels.

The determination of purified PKA activity performed in the presence or in the absence of 2 μM PAO revealed that this compound does not affect the activity of the kinase (control = 214 ± 17, PAO = 190 ± 10 pmol transferred Pi), ruling out a direct effect of PAO on the kinase itself. To study the effect of PAO on PKA activity in intact cells, MA-10 cells were stimulated with 8Br-cAMP in the

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Table 1 Measurement of cellular PKA activity in PTP inhibited MA-10 cells. Dishes of MA-10 Leydig cells (35 mm × 6 well plates) were incubated in the presence of the agents indicated in the table. Twenty micromolar H89 was used as control, since this is a well recognized PKA inhibitor. At different times, the monolayers were washed, sonicates were obtained and PKA activity was determined as described in Materials and Methods. Data express the means ± S.D. of triplicate determinations. This is a representative experiment, independently performed three times.

	PKA activity (pmol transferred Pi/mg protein)	
	15 min	60 min
Treatment		
Control	1·2 ± 0·6	4·5 ± 0·3
PAO (2 μM)	2·1 ± 0·6	3·3 ± 0·5
H89 (20 μM)	2·0 ± 0·7	2·3 ± 0·2
8Br-cAMP (1 mM)	39·0 ± 4·0*	27·0 ± 2·0*
PAO (2 μM)+8Br-cAMP (1 mM)	33·9 ± 3·7	33·6 ± 3·0
H89 (20 μM)+8Br-cAMP (1 mM)	4·0 ± 0·8†	4·2 ± 0·3†

*$P<0·001$, significant activation by 8Br-cAMP; †$P<0·001$, significant inhibition of PKA by H89 in stimulated cells.

absence or presence of PAO. After 15 min or 1 h of stimulation, endogenous PKA activity was determined. As shown in Table 1, 8Br-cAMP increases PKA activity at both times tested, and PAO was unable to inhibit this effect even when it was effective in inhibiting steroid synthesis (control=2·1 ± 0·9, 8Br-cAMP=49·0 ± 3·5, 8Br-cAMP plus PAO=7·3 ± 2·0 ng progesterone/ml, at 1 h of stimulation). In contrast, H89 (20 μM) abolished the effect of 8Br-cAMP on endogenous PKA activity (Table 1) and also on steroidogenesis (8Br-cAMP plus H89=3·2 ± 1·2 ng progesterone/ml, at 1 h of stimulation). These results indicate that PAO does not affect catalytic activity directly, nor does it affect any other event involved in PKA activation (i.e. cAMP binding or subunits dissociation).

Effects of PAO upon hCG- and 8Br-cAMP-induced StAR protein levels

To determine whether PTP activity is involved in the hormone-stimulated increase in StAR protein, we analyzed the effects of *in vivo* PTP inhibition by 2 μM PAO on hCG- or 8Br-cAMP-stimulated StAR protein levels, evaluated by Western blot. StAR protein was detected as a band with an apparent molecular mass of 30 kDa (Fig. 1A). StAR antibody also identified a faster migrating band that was present in all samples and may represent a cross-reacting protein. Analysis of either the absolute or relative intensity of the StAR signal (Fig. 1, A and C respectively) demonstrates that 1 h of stimulation with hCG or 8Br-cAMP induces StAR protein (Fig. 1A and C, lanes 2 and 3 vs lane 1) as previously demonstrated (Clark *et al.* 1995). This time period is sufficient to detect an

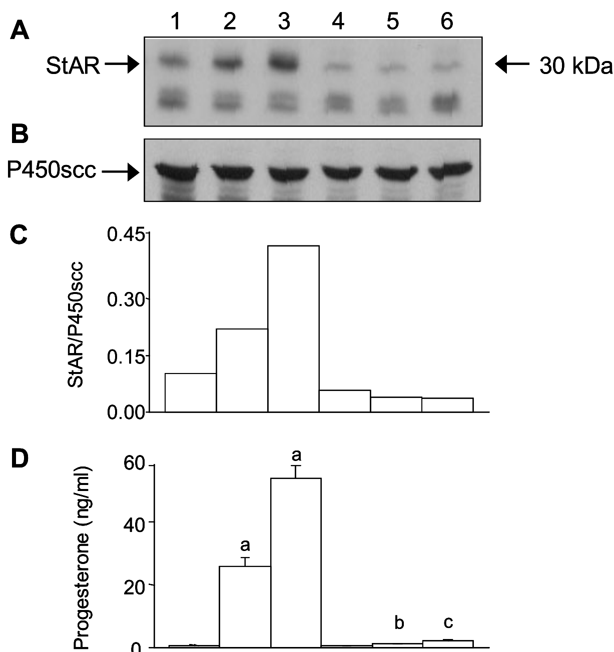

Figure 1 Effect of PAO on StAR protein induction by hCG and cAMP. MA-10 Leydig cells were incubated alone (lanes 1 and 4), with hCG (lanes 2 and 5) or 8Br-cAMP (lanes 3 and 6) in the presence (lanes 4, 5 and 6) or absence (lanes 1, 2 and 3) of 2 μM PAO. After 1 h at 36 °C, the media were removed and the cells were collected by scraping and subjected to cellular fractionation as described in Materials and Methods. Mitochondrial proteins (25–50 μg) were analyzed by Western blot with anti-StAR antibody and, after stripping the blots, with an anti-P450 scc antibody. Immunospecific bands for StAR and P450 scc proteins were quantitated by computer assisted image analysis and the data expressed as StAR/P450 scc. The figure shows the results of a representative experiment, performed three times. (A and B) Immunoblots of 30 kDa StAR protein and P450 scc respectively. (C) StAR/P450 scc ratio for each lane. (D) Steroid production of the same experiment, where the values express the means ± S.D. of the progesterone concentration registered in the three separate wells that originated the sample analyzed in panels A and B (a, hCG and 8Br-cAMP vs control, $P<0·001$; b, hCG plus PAO vs hCG, $P<0·001$; c, 8Br-cAMP plus PAO vs 8Br-cAMP, $P<0·001$).

inhibitory action of PAO on this induction, since this inhibitor notably reduces StAR protein levels induced by both hCG and cAMP analog (Fig. 1A and C, lane 5 vs lane 2, and lane 6 vs lane 3). In addition, as was previously demonstrated, PAO inhibits the steroid production stimulated by these treatments (Fig. 1D). Thus, it appears that tyrosine phosphatases activity is necessary for StAR protein expression and steroidogenesis.

Time course of PAO action on StAR protein levels

It was previously reported that StAR protein levels are hormonally increased in a time-dependent fashion in MA-10 cells, reaching a maximal level after 6 h of treatment with hCG or cAMP (Clark *et al.* 1995).

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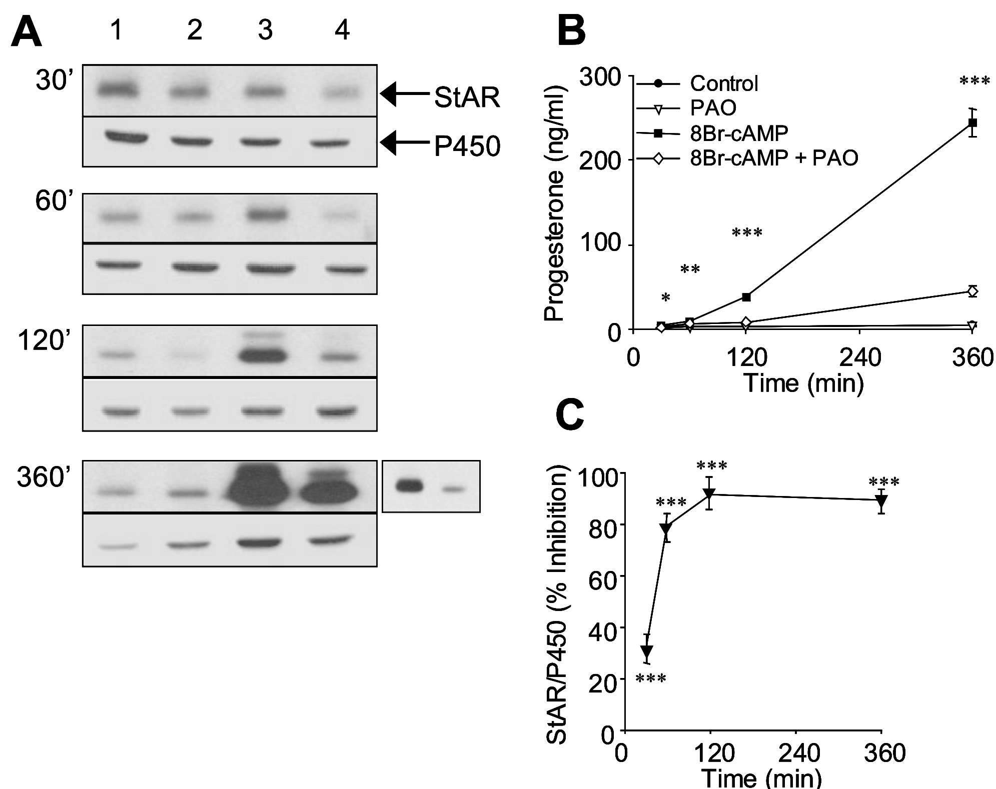

Figure 2 Time course of 8Br-cAMP-induced StAR protein levels under PTP inhibition. MA-10 Leydig cells were incubated alone (lanes 1 and 2) or with 8Br-cAMP (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2 μM PAO for the indicated times. After the incubation, the media were removed and the cells were collected by scraping and subjected to cellular fractionation as described in Materials and Methods. Mitochondrial proteins (25–50 μg) were analyzed by Western blot with an anti-StAR antibody and, after stripping the blots, with an anti-P450 scc antibody. Immunospecific bands for StAR and P450 scc proteins were quantitated by computer assisted image analysis. (A) Representative Western blot of StAR protein. A shorter exposure of the membrane corresponding to the stimulated samples at 360 min is shown on the right. (B) Steroid production of the same experiment, where the values express the mean ± S.D. of the progesterone concentration registered in the three separate wells that originated the sample analyzed in (A). The asterisks denote statistical differences between 8Br-cAMP and 8Br-cAMP plus PAO: *P<0·05; **P<0·01; ***P<0·001. These results were reproduced in a further two experiments independently performed. (C) Percentage inhibition of PAO action on 8Br-cAMP-induced StAR/P450 scc ratio, where the values express the mean ± S.D. of three independent experiments. ***P<0·001.

Therefore, in the next series of studies the profile of PAO inhibition on StAR protein levels and progesterone production was evaluated in a period between 30 and 360 min. Panels A and B in Fig. 2 show the Western blot and progesterone production of a representative experiment. A period of 30 min incubation with PAO is enough to detect its inhibitory action on 8Br-cAMP-mediated StAR protein induction in MA-10 cells (Fig. 2A, lane 4 vs lane 3). At all time points examined the amount of StAR protein in cells treated with 8Br-cAMP plus PAO is lower than the amount present in cells treated with 8Br-cAMP alone and these differences are more pronounced at prolonged incubation times (Fig. 2A, lane 4 vs lane 3). In agreement with the obligatory role of StAR in steroid synthesis, progesterone accumulation in the culture medium from 8Br-cAMP plus PAO-treated cells is lower than that registered in the medium from cells treated with 8Br-cAMP alone and this is particularly evident after long periods of incubation (Fig. 2B). However, progesterone production at 360 min in 8Br-cAMP plus PAO-treated cells does not parallel the amount of the StAR protein as it was described above for 30, 60 and 120 min. As can be seen in Fig. 2A, the amount of StAR protein at 360 min in cells treated with 8Br-cAMP plus PAO is apparently higher than the amount of StAR protein at 120 min in cells incubated in the presence of 8Br-cAMP alone (Fig. 2, 360 min, lane 4 vs 120 min, lane 3). Progesterone production, however, is not significantly higher (Fig. 2B: 360 min, 8Br-cAMP+ PAO=44·9 ± 6·8; 120 min, 8Br-cAMP=38·4 ± 3·7 ng/ml). The temporal profile of PAO

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Table 2 Measurement of total protein synthesis in PTP inhibited MA-10 cells. Dishes of MA-10 Leydig cells (35 mm × 6 well plates) were pre-incubated with 20 μCi/ml ^{35}S-methionine for 20 min and subsequently incubated in the presence of the agents indicated in the table. Cycloheximide (10 μg/ml) was used as control, since this is a well recognized protein synthesis inhibitor. At different times, the monolayers were washed extensively and homogenates were obtained as described in Materials and Methods. Total radioactive incorporation was determined by counting the radioactivity in an aliquot of the homogenates. Aliquots were precipitated with TCA, precipitated proteins were loaded onto glass fiber filters and radioactive incorporation into proteins was determined after washing the filters thoroughly. Results are the percentage of total cellular ^{35}S-methionine and are expressed as the mean ± S.D. of triplicate determinations. These results were reproduced in two other experiments independently performed

	Protein ^{35}S-Methionine/ cellular ^{35}S-methionine (%)	
	2 h	5 h
Treatment		
Control	45 ± 5	49 ± 4
PAO (2 μM)	46 ± 4	49 ± 5
8Br-cAMP (1 mM)	38 ± 3	48 ± 3
PAO (2 μM) 8Br-cAMP (1 mM)	35 ± 5	48 ± 5
Cycloheximide (10 μg/ml)	12 ± 3*	—

**P*<0·001 significant inhibition by cycloheximide compared with all treatments.

inhibition on 8Br-cAMP-induced StAR protein levels obtained in three independent experiments and expressed as percentage inhibition is shown in Fig. 2C. As can be seen, a period of 30 min of incubation with PAO is enough to produce 35% inhibition of StAR protein induction (Fig. 2C). The inhibitory effect of PAO progresses and reaches the maximal value (90% inhibition) at 120 min, remaining unchanged until 360 min (Fig. 2C).

Determination of the rate of protein synthesis in the presence of PAO

The results obtained on StAR protein levels could be explained by the fact that PAO acts through an inhibition of general protein synthesis, an observation in keeping with previous findings that hormone-stimulated steroid production has an absolute requirement for the synthesis of new proteins (Garren *et al.* 1965, Cooke *et al.* 1975). However, treatment of the cells with 2 μM PAO for 2 or 5 h did not modify the quantity of newly synthesized proteins, as evaluated by monitoring ^{35}S-methionine incorporation into proteins (Table 2).

These results indicate that the effect of PAO on stimulated steroid production and StAR protein levels is not due to a general inhibitory effect on protein synthesis. Moreover, our previous results demonstrating no inhibitory action of PAO on 22R HC-supported steroidogenesis exclude the possibility of an effect of this compound on steroidogenic enzyme activity (Cornejo Maciel *et al.* 2001).

Effect of PAO on StAR mRNA levels

Given that StAR mRNA levels in MA-10 cells are acutely induced by hormone stimulation (Clark *et al.* 1995, 1997), the effect of PAO on StAR protein content in stimulated cells could result from a decrease in StAR gene transcription. Thus, to assess whether PAO is acting at this step, the time course of StAR mRNA levels in MA-10 cells stimulated with 8Br-cAMP in the presence or absence of 2 μM PAO was determined. Northern blot analysis revealed two major bands of StAR mRNA at 1·6 and 3·4 kilobase pairs in 8Br-cAMP-treated cells (Fig. 3A, lane 3) as previously described (Clark *et al.* 1995). cAMP analog elicits a time-dependent increase in both transcripts (Fig. 3A, lane 3) and PAO treatment impairs this increase (Fig. 3A, lane 4 vs lane 3). Quantitation of the StAR mRNA signals obtained in the Northern blots indicates that incubation of the cells with PAO for as short as 60 min is sufficient to produce an 85% inhibition in 8Br-cAMP-induced StAR mRNA levels (Fig. 3B). Even though longer periods of incubation produce a somewhat weaker effect, PAO still retains its inhibitory capacity after 360 min of treatment (Fig. 3).

Discussion

Protein phosphatase activity is involved in diverse cellular processes. Given that the signaling cascade triggered by steroidogenic tropic hormones is mediated by Ser/Thr kinase activation, the studies on the role played by protein dephosphorylation were initially focused on Ser/Thr phosphatases (PPs) (Iyer *et al.* 1988, Azhar *et al.* 1991, Sayed *et al.* 1998, Jones *et al.* 2000). Several recent reports indicate that tyrosine dephosphorylation is an active component of the steroidogenic pathway (Vilgrain et at 1998, Paz *et al.* 1999, Cornejo Maciel *et al.* 2001, Sewer & Waterman 2002), and the present study provides evidence that PTP activity participates in the mechanism by which LH/CG and 8Br-cAMP increase StAR protein levels. This conclusion is supported by the fact that the increase in the levels of both StAR protein and mRNA caused by tropic hormone or by the second messenger, 8Br-cAMP, is partially inhibited by PAO, a PTP inhibitor. This inhibition occurs at a concentration that reduces hCG- and 8Br-cAMP-stimulated steroid production but does not modify basal or 22R HC-supported steroidogenesis, clearly showing that the effect is not due to an inhibition of P450 scc activity. These results are also in agreement with previous observations suggesting that the site of PAO action was located between PKA activation and cholesterol transport across the mitochondrial membrane.

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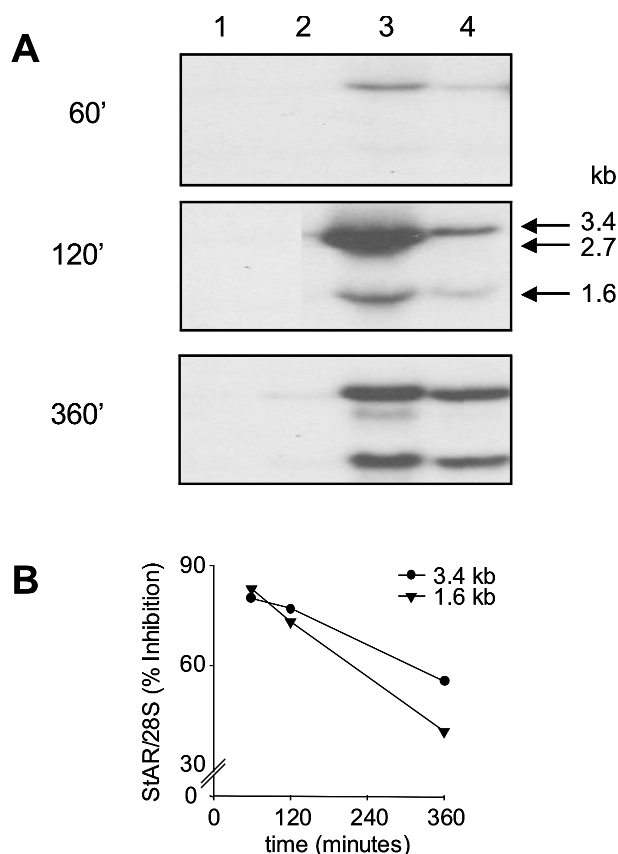

Figure 3 Time course of 8Br-cAMP-induced StAR mRNA levels under PTP inhibition. MA-10 Leydig cells were incubated alone (lanes 1 and 2) or with 8Br-cAMP (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2 µM PAO for the indicated times. Total cellular RNA was isolated and analyzed by Northern blot with specific probes to StAR mRNA and 28S rRNA as described in Materials and Methods. Specific bands were quantitated by computer assisted image analysis. (A) Representative Northern blot for StAR mRNA. (B) Percentage inhibition produced by PAO on the 3·4 and 1·6 transcripts of StAR mRNA/18S rRNA ratios. These results were reproduced in a further two experiments independently performed.

On the basis of our previous observations (Paz *et al.* 1999, Cornejo Maciel *et al.* 2001), we hypothesized that PTP activity could be involved in the regulation of steroidogenesis by tropic hormones through the regulation of PKA activity or StAR protein synthesis. The action of PAO on PKA activity is clearly ruled out since the compound inhibits neither the dissociation nor the activity of the enzyme, showing that 8Br-cAMP can activate PKA in the absence or presence of PAO. Thus, these results constrain even more the site of action of PAO and reinforce the possibility that its action on steroidogenesis targets StAR protein induction. This theory is supported by the results obtained in the present study where inhibition of PTP activity by PAO reduces the effects of 8Br-cAMP on StAR protein levels, indicating that PTPs are involved in regulating the quantity of this protein that is essential for cholesterol delivery into the mitochondria.

Moreover, the inhibition of StAR protein level detected by immunoblot follows the inhibition on progesterone production. An unexpected observation was that at 360 min of PAO+8Br-cAMP treatment there is still a marked reduction in progesterone production in the presence of StAR levels which are even higher than the amount of StAR present at shorter times in the absence of PAO. This suggests that the increase in StAR protein in the presence of 8Br-cAMP observed between 120 and 360 min is not related to the increase observed in progesterone production in the same period. Nevertheless, our results indicate that one of the targets of PTP inhibition is at the level of StAR protein synthesis, although we cannot rule out that PTP may act in other specific steps of the steroidogenic cascade after prolonged exposure to the stimuli. The specificity of this action was demonstrated by the fact that PAO does not affect other events required for steroidogenesis, i.e. protein synthesis, PKA activity or steroidogenic enzyme activities.

Another possible explanation for the lower levels of StAR protein in PAO-treated cells would indicate an action of this compound on StAR protein degradation. PAO somewhat diminished StAR basal expression, however the magnitude of this effect was less than on stimulated StAR protein expression and remained unchanged during the time tested (30 – 360 min). The fact that the inhibitory effect of PAO is more evident in stimulated cells suggests that PAO is acting on StAR protein synthesis rather than on its stability.

StAR mRNA levels were also reduced by inhibition of PTP activity. However, while PAO reduces StAR protein as well as mRNA levels, the profiles of inhibition are different. The inhibitory effect of 8Br-cAMP on StAR protein levels is maximal at 120 min (90%) and stays constant until 360 min, while the major action of PAO upon StAR mRNA is observed in a shorter period of time (60 min) and appears to be transient as longer periods of time reduce this effect.

Although PTP activity is involved in the control of several processes (Neel & Tonks 1997, Tonks & Neel 2001), at this time it is difficult to explain how these enzymes could be regulating induced StAR protein levels. The effect of PAO on StAR mRNA transcription might suggest that PTP activity is required to activate a transcription factor involved in this process. However, while the activation of transcription factors by phosphorylation in tyrosine residues is well documented (Shuai *et al.* 1992, Cho *et al.* 1996), the modulation of the transcription process by PTP is still poorly described (Wang *et al.* 1997, Servidei *et al.* 1998). A transcription process that involves tyrosine as well as serine/threonine dephosphorylation emerges from a recent publication regarding the transcription of CYP17 gene (Sewer & Waterman 2002), the product of which is an enzyme that participates in the

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steroid synthesis. Interestingly, the transcription of this gene requires the activity of SF-1, a transcription factor also involved in StAR gene expression (Reinhart *et al.* 1999). Using inhibitors of Ser/Thr and Tyr phosphatases, the authors found an inhibition of cAMP-inducible binding of SF-1, among other transcription factors, to the promoter of CYP17, and a concomitant reduction in its activity.

Our results and several previous studies (Iyer *et al.* 1988, Sayed *et al.* 1998, Azhar *et al.* 1991, Jones *et al.* 2000, Sewer & Waterman 2002) show that the activity of both serine/threonine and tyrosine phosphatases is involved in the stimulation of steroid synthesis. Since dual specificity phosphatases were described, it is possible to speculate that a phosphatase of this kind could be acting on the hormonal regulation of steroidogenesis.

In summary, our data support the involvement of PTPs on hormone-regulated StAR protein levels. Our hypothesis is that a phosphotyrosine protein exerts a negative control on steroidogenesis. Tyrosine dephosphorylation of this protein by hormone-activated PTP would abolish its inhibitory effect, allowing the induction of StAR protein. Given that PTP activation by LH/CG, as well as by ACTH, has already been demonstrated, our findings highlight the critical role of hormonally regulated PTPs on LH/CG-stimulated StAR protein induction and, consequently, upon the hormone-stimulated steroidogenesis.

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