Pergamon

0960-0760(95)00059-3

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 1/2, pp. 31–38, 1995

0960-0760/95 $9.50 + 0.00

Regulation of Guinea Pig Adrenal *P*450c21 Messenger RNA, Protein and Activity by RU486

Michel Vallée, Sylvie Perron, Yves Tremblay* and Alain Bélanger†

MRC Group in Molecular Endocrinology, CHUL Research Center and Laval University, Centre Hospitalier de l'Université Laval, Quebec, Canada G1V 4G2

The role of ACTH, forskolin and 8Br-cAMP on the regulation of mRNA abundance, protein levels and enzymatic activity of cytochrome *P*450 21-hydroxylase (*P*450c21, CYP21) were investigated in guinea pig adrenal cell cultures. In untreated cells, 21-hydroxylase activity was diminished throughout a 48 h period of incubation. Although incubation with forskolin and 8Br-cAMP restored 21-hydroxylase activity to normal levels, the addition of ACTH did not prevent the decrease of 21-hydroxylase activity. Treatment of cells with RU486 for 24 h inhibited 21-hydroxylase activity by 93%; however, after removal of the drug a slight increase of enzyme activity was observed; this rise was enhanced by the addition of ACTH. Forskolin and 8Br-cAMP increased the levels of 21-hydroxylase activity to the same range as seen in untreated cells. In cells that were not pretreated with RU486, incubation with cycloheximide for 1 h had no effect on 21-hydroxylase activity and could not prevent the modest increase of 21-hydroxylase activity induced by forskolin or 8Br-cAMP after 48 h of incubation. In RU486-treated cells, cycloheximide blocks the stimulation of enzyme activity induced by ACTH, forskolin and 8Br-cAMP. Our findings indicate that 21-hydroxylase activity can be stimulted by ACTH, forskolin or 8Br-cAMP solely in the presence of reduced enzymatic activity. Western immunoblot analysis of *P*450c21 protein levels in untreated or RU486-treated adrenal cells indicate that *P*450c21 protein levels were in the same range and further incubation with ACTH caused a similar elevation of *P*450c21 protein levels in both the untreated and RU486-treated cells. Northern blot analysis on RNA isolated from adrenal cells showed that RU486 did not alter the basal steady state levels of *P*450c21 mRNA. As well, incubation with ACTH or 8Br-cAMP increased the levels of *P*450c21 transcript to the same extent in both untreated and RU486-treated cells. These results taken together provide additional evidence for the presence of an adrenal specific protein factor(s) modulating 21-hydroxylase activity.

J. Steroid Biochem. Molec. Biol., Vol. 54, No. 1/2, pp. 31–38, 1995

INTRODUCTION

Cytochrome *P*450 21-hydroxylase (*P*450c21, CYP21) is a steroidogenic enzyme expressed exclusively in the adrenals that converts progesterone to 11-deoxycorticosterone and 17-hydroxyprogesterone to 11-deoxycortisol [1]. Transfection of the cloned murine *P*450c21 cDNA into the mouse Y1 adrenocortical tumor cell line led to expression of *P*450c21 mRNA and 21-hydroxylase enzymatic activity; the expression was further stimulated by ACTH which suggests that ACTH plays a predominant role as regulator of the CYP21A gene expression [2]. Although these observations indicate that a direct relationship between protein levels and enzymatic activity controlled by ACTH might exist, the existence of other factors involved in the regulation of 21-hydroxylase activity has also been suggested [3]. Using bovine adrenal cells in culture, it was previously shown that ACTH stimulates gene expression, protein synthesis and enzymatic activity of cytochromes *P*450scc, *P*450c17 and *P*450c11 [4] while there was no effect on 21-hydroxylase activity in the presence of a marked increase of *P*450c21 protein levels [3]. Chang *et al.* [5] recently reported that, in bovine cell cultures, the response of *P*450c21 mRNA and 21-hydroxylase activity to cAMP

*Holder of a scholarship from the Fonds de la Recherche en Santé du Québec (F.R.S.Q.).
†Correspondence to A. Bélanger.
Received 15 Sep. 1994; accepted 6 Jan. 1995.

depends on the conditions under which bovine adrenocortical cells are cultured, thus also suggesting the existence of controlling factors for *P*450c21 gene expression other than cAMP. Previous studies by our group also showed that ACTH was unable to stimulate 21-hydroxylase activity despite the 25-fold stimulation of *P*450c21 mRNA abundance [6].

C19 steroids have been found to interact with several steroidogenic enzymes and alter their activity [7]. Using bovine adrenal cells, Hornsby *et al.* [8,9] demonstrated an inhibition of 11β-hydroxylase and 21-hydroxylase activities after incubation with androstenedione. Decreases of Leydig cells *P*450c17 protein levels and 17-hydroxylase enzyme activity by testosterone were also reported by Perkins *et al.* [10] showing that the alteration of steroidogenic enzymes by C19 steroids was not limited to the adrenals. It has been proposed that the alteration of adrenal 11β-hydroxylase and testicular 17α-hydroxylase occurs through the same mechanism, e.g. binding of C19 steroids to the steroidogenic enzyme as well as the enhancement of the generation of oxygen-derived radicals which directly or indirectly inactivate the enzyme [11]. However, the mechanism involved in the inhibition of 21-hydroxylase activity is much less understood. Androstenedione also causes, in guinea pig adrenal cells, an inhibition of 21-hydroxylase and 11β-hydroxylase activities, while it was shown that the reduction in oxygen from 19 to 2% in guinea pig cells partially prevents the decrease of 11β-hydroxylase activity caused by androstenedione but failed to protect 21-hydroxylase activity, thus suggesting that the formation of oxygen-derived radicals is not involved in the reduction of 21-hydroxylase activity [12]. In addition, Hornsby [13] also observed that 11β-hydroxylase activity in bovine adrenal cells may be more efficiently protected by concomitant treatment with antioxidants which reduce peroxide formation. It must also be mentioned that, in contradiction to the inhibition of 17α-hydroxylase activity by C19 steroids observed in Leydig cells, a marked stimulation of 17α-hydroxylase and 17,20-lyase activities was found in guinea pig adrenal cells incubated with androstenedione [12]. In fact, a marked increase of 17α-hydroxylase and 17,20-lyase activities occurs concomitantly with the decrease of guinea pig adrenal 21-hydroxylase activity in guinea pig adrenal cells treated with RU486, a synthetic C18 steroid having a 4-ene-3-ketosteroid with an aryl group at position 11 [14].

In the present study, the role of ACTH, forskolin and 8Br-cAMP in the regulation of mRNA abundance, protein synthesis and enzymatic activity of the cytochrome *P*450c21 enzyme was investigated in guinea pig adrenal cell cultures previously treated with RU486. Our data show that guinea pig adrenal 21-hydroxylase activity could not be increased by ACTH although *P*450c21 mRNA and protein levels were elevated. When cells were treated with RU486, there was inhibition in 21-hydroxylase activity. The addition of ACTH to RU486-treated cells caused a marked increase of 21-hydroxylase activity accompanied by a stimulation of *P*450c21 mRNA and protein levels to the values seen in non-treated cells.

MATERIALS AND METHODS

Chemicals

Trypsin inhibitor (from soybean), trypsin, deoxyribonuclease 1 (from bovine pancreas) and collagenase (from *Clostridium histolyticum*) were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Eagle's minimum essential medium (MEM), bovine serum albumin (BSA; fraction V), Hanks' balanced salt solution and HEPES (*N*-2-hydroxyethylpiperazine *N*′2-ethanesulphonic acid) were obtained from Gibco (Grand Island, NY, U.S.A.). Fetal calf serum was obtained from Hyclone Laboratories (Logan, UT, U.S.A.) and 24-well culture plates from Flow Laboratories (McLean, VA, U.S.A.). The synthetic tetracosapeptide ACTH (Cortrosyn) was supplied by Organon (West Orange, NJ, U.S.A.). 8-bromoadenosine 3′,5′-cyclic monophosphate (8Br-cAMP), forskolin, cycloheximide and all of the chemicals used for mRNA preparation and analysis were purchased from Sigma Chemicals Co. (St Louis, MO, U.S.A.) with the exception of guanidine thiocyanate which was obtained from Clonetech Inc. (Palo Alto, CA, U.S.A.). RU486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(prop-1-ynyl)-estra-4,9-dien-3-one) was obtained from Roussel UCLAF (Paris, France). [1,2,6,7-^{3}H]17-hydroxyprogesterone (74 Ci/mmol) was obtained from Amersham (Oakville, Ontario, Canada). Unlabelled 17-hydroxyprogesterone was purchased from Steraloids Inc. (Wilton, NH, U.S.A.).

Preparation of dispersed adrenal cells

Adult male guinea pigs (Hartley), weighing approx. 900 g, obtained from Charles River Canada Inc. (St Constant, Quebec, Canada) were used for the preparation of primary culture of adrenal cells. Animals were decapitated and adrenals were quickly removed using sterile instruments, trimmed of fat and placed in cold Hanks' solution. The adrenal cells were isolated using an adaptation of the procedure described by Black *et al.* [15] and modified as previously reported [16]. Adrenal cells were plated at a density of 1.25×10^6 cells/ml in 10 ml MEMS in 100 × 20 mm petri dishes for the measurement of mRNA and protein levels, and in 24-well petri dishes in 1 ml MEMS at a density of 2.5×10^5 adrenal cells/ml in order to determine enzymatic activity. Cells were placed under 5% carbon dioxide in humidified air at 37°C and were used 24 h after plating. Treatment with various compounds was initiated for the indicated periods of time. RU486 was added from ethanolic stock solution and the final

ethanol concentration was 0.01% (v/v) in all treatment media.

Enzymatic activity

At the end of the period of incubation, medium was then removed and cells were incubated with MEM alone for 10 min to remove steroids. The enzyme activity was then assayed as follows. Tritiated 17α-hydroxyprogesterone (0.36 μCi) and 17α-hydroxyprogesterone (5×10^{-6} M) were incubated for 120 min and values, in duplicate, for *P*450c21 activity were obtained by calculating conversion of 17-hydroxyprogesterone into deoxycortisol and cortisol. The enzymatic reaction was stopped by adding 75 μl acetic acid (0.5 M) to the culture wells, the medium was recovered and frozen at −20°C until assayed. The products of metabolism were assayed as follows. The medium was extracted twice with 5 ml of ethyl ether:acetone (1:1, v/v) and the extract was evaporated to dryness. The remaining residue was dissolved in a mixture of methanol:water (1:1, v/v) and subjected to high-performance liquid chromatography (HPLC) analysis using a Waters model 510 chromatograph (Waters Instruments, Milford, MA, U.S.A.) and a C18 column (Radial-Pak, Waters Instruments). A gradient of 100% methanol–water (1:1, v/v) to 41% tetrahydrofuran–acetonitrile (1:1, v/v) over a 35 min period was used for the analysis of 17α-hydroxyprogesterone metabolism. Radioactive compounds were detected with a Berthold model LB 506 HPLC radioactivity monitor (Weldbrod, Germany) and subsequently integrated using a Waters 740 single-channel recorder/integrator (Waters Associates, Milford, MA). The formation of tritiated metabolites was calculated by dividing the amount of radioactivity in the product peak by the sum of the radioactivity in all peaks recovered after chromatography, thus obtaining the percentage of substrate converted to that product. Enzyme activity was expressed in pmol metabolites formed by 10^6 cells per hour. The rate of conversion of steroid substrate was linear for the times indicated.

mRNA preparation and analysis (Northern blot analysis)

At the end of the period of incubation, adrenal cells were harvested and collected by centrifugation at 200 *g* for 5 min at 4°C and resuspended in 900 ml of 4 M guanidine thiocyanate, 0.1 M 2-mercaptoethanol, 2% *N*-laurylsarcosine, 0.05 M Tris–HCl (pH 7.5) and 0.05 M EDTA. Cellular RNA was obtained by centrifugation at 120,000 *g* for 16 h at 20 C through 5.7 M CsCl [17]. The RNA pellets obtained were dissolved in 0.5% sodium dodecyl sulphate (SDS) and 0.01 M Tris–HCl (pH 7.5), and then precipitated twice with ethanol.

Northern blots were performed as previously described [18]. Briefly, 25 μg of total mRNA was denaturated in glyoxal [19], electrophoresed, transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL, U.S.A.) in 20 × SCC [1 × SCC = 0.150 M NaCl, 0.015 M sodium citrate (pH 7.0)] overnight at 25°C. RNA was immobilized covalently by cross-linking under short wavelength UV for 1 min. Hybridization was performed under high-stringency conditions as previously described [19]. Filters were washed in 0.1 × SSC with 1% SDS for 20 min at room temperature, followed by three 20 min washes in the same solution at 65°C.

For RNA dot blot hybridization assays, RNA was denaturated by heating at 65°C for 15 min in 6 × SCC containing 7.5% formaldehyde. Two-fold serial dilutions of RNA in 10 × SCC were blotted onto nylon membranes using a 96-well Hybri-dot Manifold (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). All dots for each experiment were on a single filter. Dot blots were processed as described above for Northern blots. The Northern and dot blots were then autoradiographed at −80°C with intensifying screens.

The intensities of the autoradiographic spots were quantified using an Amersham RAS Image Analyzer system. The slopes of the dot intensities of each dilution series were calculated by linear regression using Cricket Graph (Cricket Software, Malvern, PA, U.S.A.).

Probes

The *P*450c21 probe was a 1200 bp EcoR1 fragment of human *P*450c21 cDNA [20]. The probe was labeled with [α-^{32}P]dCTP to a specific activity of 10^9·cpm/μg using a random primer kit purchased from Amersham. An oligonucleotide probe [21] specific for rat 18S ribosomal RNA was synthesized (Bioresearch DNA synthesizer) and end-labeled with [γ ^{32}P]ATP and used to assess the amount and the integrity of RNA loaded.

Protein extraction and immunoblot analysis

Protein was isolated from guinea pig adrenal tissue cell cultures using the Tri Reagent protocol according to the manufacturer (Molecular Research Center, Cincinnati, OH, U.S.A.). 50 μg of protein was fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, treated with anti-rabbit *P*450c21, and exposed to a horseradish-peroxidase-labeled donkey anti-rabbit immunoglobulin G. Rabbit antibodies against *P*450c21 were developed in collaboration with Dr Walter L. Miller of San Francisco, who purified the truncated human *P*450c21 protein obtained from overexpression in *E. coli*. This truncated *P*450c21 protein (38 kDa) does not contain the amino portion of the enzyme. The antibody revealed a unique band migrating at 50,000 Da, and the specificity of the antiserum was tested against purified *P*450c21 protein and guinea pig adrenal purified microsomes. The antigen–antibody complex was detected by enhanced chemiluminescence

after the addition of luminol and hydrogen peroxide (ECL, Amersham, Arlington Heights, IL, U.S.A.).

Data analyses

Data presented are the mean ± SD of duplicate determinations. Each experiment was repeated three times and similar results were always obtained; the results shown are those of one representative experiment.

RESULTS

The effects of ACTH, an activator of adenylate cyclase or 8Br-cAMP, a cAMP agonist on basal and RU486-treated adrenal cells are shown in Fig. 1. In cells that were not treated with RU486, the levels of 21-hydroxylase activity decreased throughout a 48 h period of incubation. Treating the cells with ACTH for the same 48 h did not avoid the decrease of enzyme activity. However, the presence of forskolin or 8Br-cAMP restored 21-hydroxylase activity to normal levels. When the cells were treated with RU486 for 24 h, there was a decrease of 21-hydroxylase activity by 93% and this inhibitory effect remained essentially unchanged throughout the subsequent 48 h period of incubation in the presence of RU486. When RU486 was removed after the initial treatment of 24 h, the 21-hydroxylase activity was increased from 1120 ± 912 to 4325 ± 895 pmol/10^6 cells per hour over a 48 h period. The enzyme activity was further increased to 3354 ± 455 and 8226 ± 585 pmol/10^6 cells per hour in the presence of ACTH for an incubation period of 24 and 48 h, respectively. Only forskolin and 8Br-cAMP increased the levels of 21-hydroxylase activity to the range seen in cells not treated with RU486.

We then studied whether *de novo* protein synthesis was required for the induction of 21-hydroxylase activity by ACTH, forskolin or 8Br-CAMP. Adrenal cells were first treated with RU486 for 24 h, after which 50 μg/ml of cycloheximide, a protein synthesis

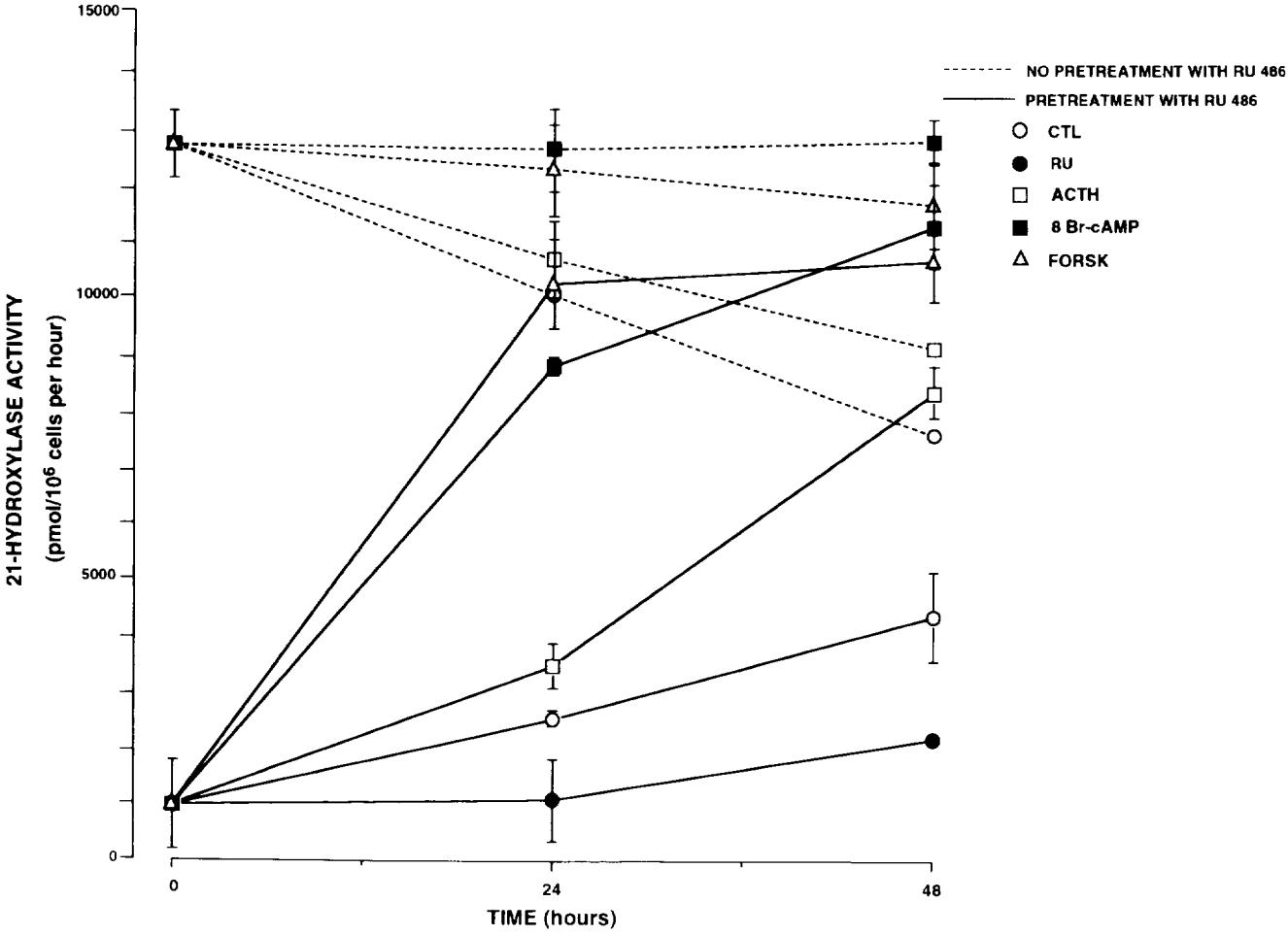


Fig. 1. Effects of ACTH (10 nM), 8-bromoadenosine 3′,5′-cyclic monophosphate (8Br-cAMP) (1 mM) and forskolin (FORSK) (1 μM) on 21-hydroxylase activity in non-treated (----) and RU486-treated (—) cells. Adrenal cells were preincubated for 24 h in the absence or presence of RU486 (1 μM). Medium was changed and cells were incubated for 24 or 48 h in the presence of the appropriate chemical product. Results are expressed in pmol of metabolites form/10^6 cells/h. Each measurement was done in duplicate and each point represents the mean ± SD.

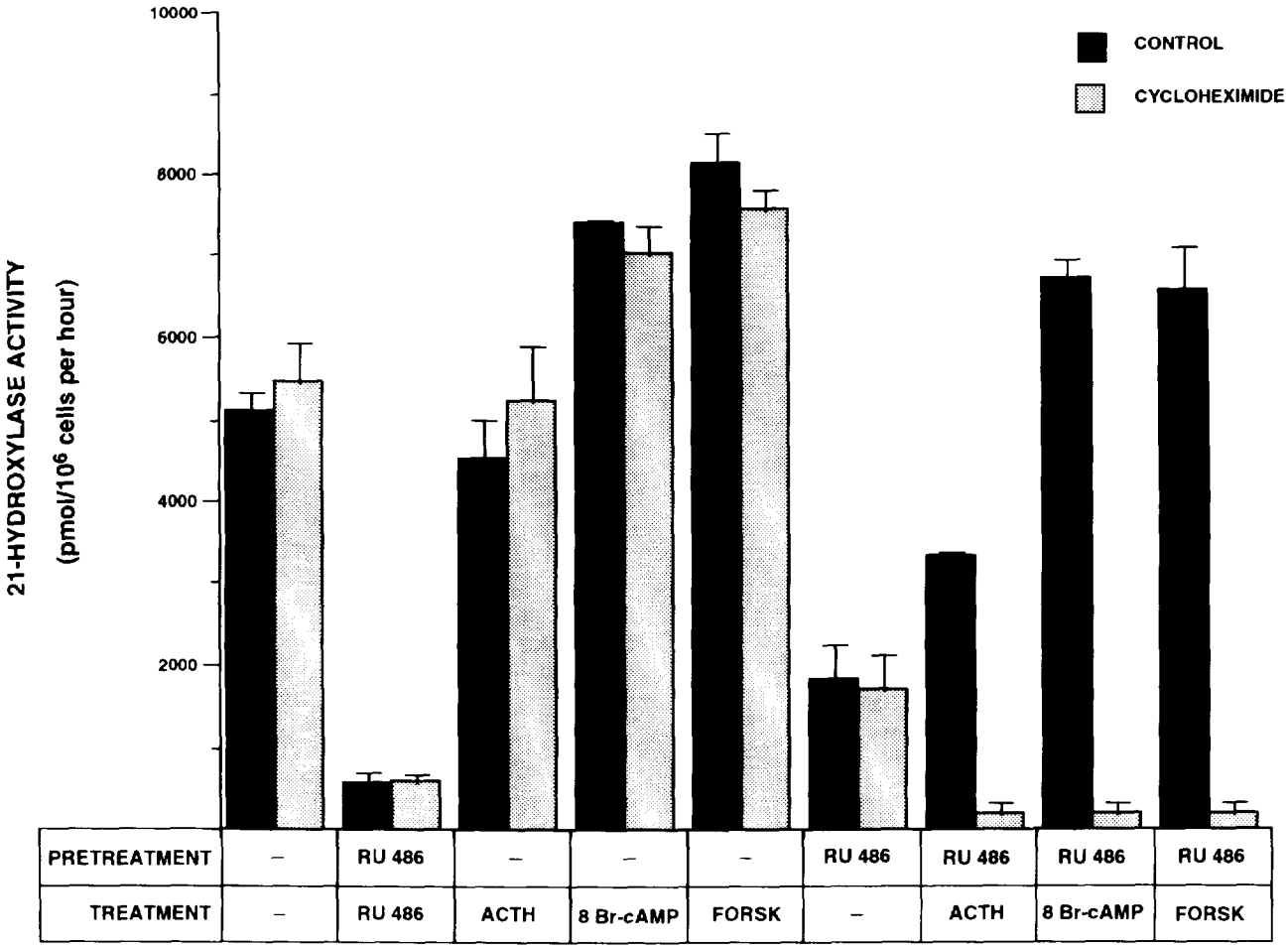


Fig. 2. Effects of cycloheximide after a 24 h pretreatment with RU486 (1 μM) and treatment with ACTH (10 nM), 8Br-cAMP (1 mM) or forskolin (1 μM) on 21-hydroxylase activity. In adrenal cells pretreated for 24 h with RU486, cycloheximide (50 μg/ml) alone was added for 1 h and then treated with cycloheximide in combination with ACTH, 8Br-cAMP or forskolin for another 4 h. Cells were washed and further incubated in the presence of the appropriate product alone for 24 h. In control plates, cycloheximide was added for 5 h before incubation with ACTH, 8Br-cAMP or forskolin. Each measurement was done in duplicate and each point represents the mean $\pm$ SD.

inhibitor, was added for 1 h and then treated with cycloheximide in combination with ACTH, 8Br-cAMP or forskolin for another 4 h and finally cells were incubated with or without ACTH, forskolin or 8Br-cAMP for 24 h. As illustrated in Fig. 2, cycloheximide had no effect on 21-hydroxylase activity either in untreated or in RU486-treated adrenal cells. Although cycloheximide could not prevent the slight increase of 21-hydroxylase activity induced by forskolin or 8Br-cAMP in untreated cells, it was capable of blocking the stimulation of 21-hydroxylase activity induced by ACTH, forskolin or 8Br-cAMP in RU486-pretreated cells.

To determine the levels of *P*450c21 protein expressed in the non-pretreated and RU486-treated guinea pig adrenal cells, we performed Western blot analysis using the cells in culture (Fig. 3). Treatment of cells with RU486 alone had no effect on *P*450c21 protein levels. In non-pretreated cells, a marked stimulation of *P*450c21 protein level was observed in the presence of ACTH and a similar increase was also observed in cells pretreated with RU486. In cycloheximide-treated cells, there was no further increase of *P*450c21 protein levels induced by ACTH both in untreated and RU486-treated cells (data not shown).

To determine the effects of ACTH and 8Br-cAMP on the steady state levels of *P*450c21 mRNA in guinea pig adrenal cells, we performed Northern blot analysis. In cells which were not pretreated with RU486, a subsequent incubation with ACTH increased the levels of *P*450c21 transcript by 9-fold while treatment with 8Br-cAMP lead to a 15-fold induction. A similar response to ACTH and 8Br-cAMP was observed when a parallel experiment using cells pretreated with RU486 was performed. As well, prolonged treatment or pretreatment with just RU486 did not alter the levels of *P*450c21 mRNA when compared to the untreated

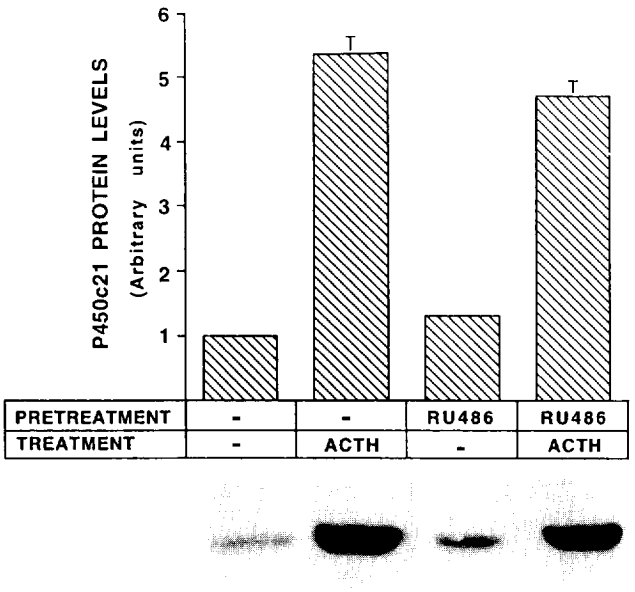


Fig. 3. Effects of ACTH (10 nM) on *P*450c21 protein levels in adrenal cells pretreated with RU486. Adrenal cells were pretreated for 24 h with RU486 (1 μM). Medium was changed and adrenal cells were incubated for 48 h in the presence of ACTH. Cells were recovered and protein was analysed as described in Materials and Methods.

control. It is thus apparent that RU486 does not alter the levels of *P*450c21 transcript.

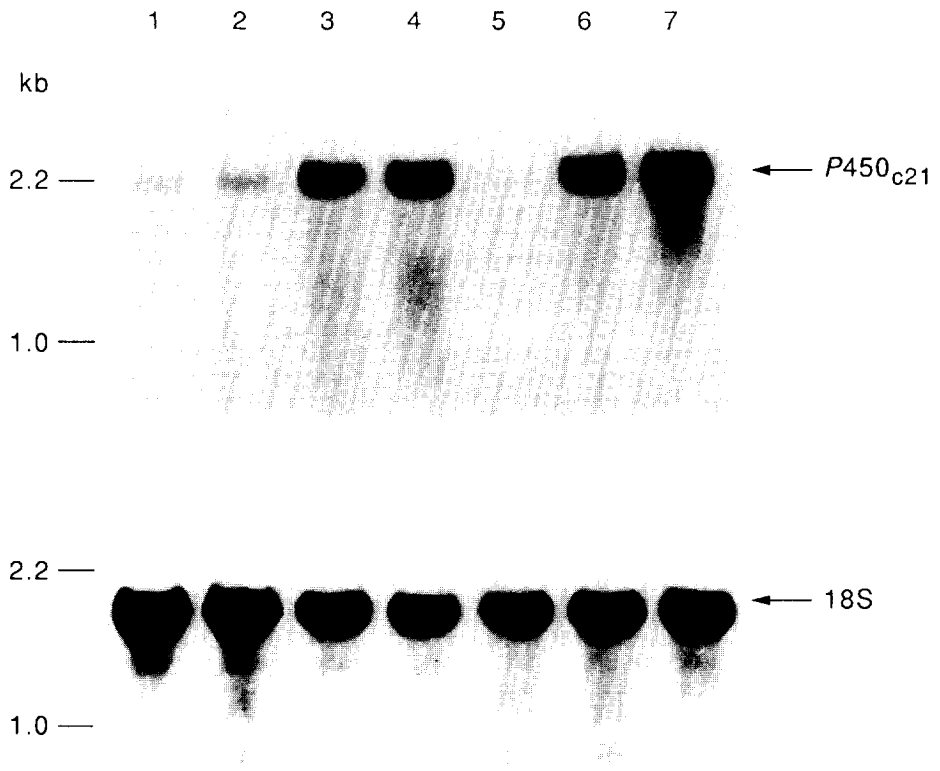
DISCUSSION

It is well established that ACTH elicits a chronic response of various steroid hydroxylase enzymes in the adrenals. Its effects on gene expression encoding the cytochromes *P*450ssc, *P*450c17, *P*450c21 and *P*450c11 concomitantly with protein synthesis have been clearly demonstrated using several *in vitro* models. It is generally believed that the corresponding enzyme activity would also increase. However, in studies performed *in vitro* using bovine adrenal cells [3] and *in vivo* in the rabbit [22–24], 21-hydroxylase activity remained unaltered by ACTH despite an elevation of approx. 2–3-fold in *P*450c21 protein synthesis. In agreement with those results, we now report that guinea pig adrenal 21-hydroxylase activity was not increased by ACTH although *P*450c21 mRNA and protein levels were elevated. Together, these observations suggest that an additional intra-adrenal factor(s) may be essential for the activation of 21-hydroxylase activity. The proposal that cellular activation of hydroxylase enzymes is influenced by a protein is not unique to *P*450c21. As an example, it has been reported that binding of lipoxygenase to a membrane-bound 18 kDa protein, called 5-lipoxygenase-activating protein (FLAP), was necessary for its activation [25]. Furthermore, a synthetic product that prevents the membrane association of FLAP and lipoxygenase was also identified [25].

The present study demonstrates that 21-hydroxylase activity is not stimulated by ACTH, although *P*450c21 mRNA abundance and protein synthesis were increased by more than 20- and 5-fold, respectively. Forskolin, an activator of adenylate cyclase, and 8Br-cAMP, an agonist of cAMP, also produced approximately the same stimulation of *P*450c21 mRNA and protein levels (data not shown). Although the treatments with forskolin and 8Br-cAMP prevent the decrease in 21-hydroxylase activity observed in untreated or ACTH-treated cells, the results obtained with concomitant incubation with cycloheximide indicate that the mechanism by which intracellular 21-hydroxylase activity was maintained does not involve *de novo* protein synthesis. However, this was not the case in RU486-treated cells where the marked stimulation of 21-hydroxylase activity, observed after addition of ACTH, forskolin and 8Br-cAMP, was completely prevented by cycloheximide. These observations indicate that new *P*450c21 protein synthesis is absolutely required to restore 21-hydroxylase activity in RU486-treated cells. This is in agreement with data indicating that in cells pretreated with RU486 the *P*450c21 protein levels were increased in response to ACTH.

The mechanism by which RU486 inhibits 21-hydroxylase activity likely occurs by direct interaction between the drug and the *P*450c21 protein. Previous studies [7–9,12] demonstrated that C19 steroids, namely androstenedione, testosterone and synthetic RU486 [14] interact with the protein *P*450c21 causing a marked inhibition of enzymatic activity. We have also shown that neither the androgen receptor nor the alteration of CYP21 gene expression is involved in the mechanism of inhibition by C19 steroids [12]. In addition, we demonstrate that the anti-glucocorticoid or the anti-progesterone properties of RU486 as well as the regulation of CYP21 gene expression are not involved in repression of 21-hydroxylase activity by RU486. Furthermore, we have shown, for the first time, that the inhibition of 21-hydroxylase activity induced by RU486 is irreversible and the return of enzymatic activity requires conditions that involve *de novo* protein synthesis. Taken together, these results strongly suggest that RU486 probably interacts with the *P*450c21 protein itself and irreversibly inhibits its enzymatic activity by preventing the *P*450c21 protein becoming enzymatically activated. Our data, as well as those of other groups, also indicate that an excess of the *P*450c21 protein has no effect on 21-hydroxylase activity [3], thus suggesting that the amount of *P*450c21 protein is not limiting. Using the activation of lipoxygenase by FLAP as a model [25], our data suggest the presence of an intra-adrenal factor where the interaction of *P*450c21 protein to this factor is a requirement for its activation.

It is also important to mention that concomitantly with the decrease of 21-hydroxylase activity induced by treatment of guinea pig adrenal cells with RU486,



Pretreatment	–	Ru	–	–	Ru	Ru	Ru	
Treatment	–	Ru	ACTH	8 Br cAMP	–	ACTH	8 Br cAMP	
$\bar{X}$	1.0	1.5	9.2	15	0.8	8	15	$n=3$

Fig. 4. Effects of ACTH (10 nM) and 8Br-cAMP on *P*450c21 mRNA levels in adrenal cells pretreated with RU486. Adrenal cells were pretreated for 24 h with RU486 (1 μM). Medium was changed and adrenal cells were incubated for 24 h in the presence of the appropriate chemicals. Cells were then recovered and mRNAs were analysed as described in Materials and Methods.

there was a stimulation of 17α-hydroxylase and 17,20-lyase activity and, in addition, the ratio between 17α-hydroxylase and 17,20-lyase activities was increased [14]. Recently, the cotransfection of *P*450c17 and *P*450 reductase cDNAs in COS-1 cells indicates that an increase in *P*450 reductase levels stimulates 17,20-lyase activity by 3-fold without altering 17α-hydroxylase activity. By contrast, an increase expression of cytochrome b5 protein by cotransfection of *P*450c17 and cytochrome b5 cDNAs could not enhance 17,20-lyase activity [26]. These results suggest that availability of *P*450 reductase may alter one of the two enzymatic conversions of *P*450c17 activity. Unfortunately, studies with cells cotransfected with *P*450c21 cDNA and other *P*450 enzymes have not been performed yet. A better understanding of the relationship between *P*450c21 protein and cofactors present in the adrenal is important for the elucidation of the mechanism that regulates 21-hydroxylase activity. This issue is currently under investigation in our laboratory.

REFERENCES

1. Miller W. L.: Molecular biology of steroid hormone synthesis. *Endocrine Rev.* 9 (1988) 295–318.
2. Parker K. L., Chaplin D. D., Wong M., Seidman J. G., Smith J. A. and Shimmer B. P.: Expression of murine 21-hydroxylase in mouse adrenal glands and in transfected Y1 andrenocortical tumor cells. *Proc. Natn. Acad. Sci. U.S.A.* 82 (1985) 7860–7864.
3. Funkenstein B., McCarthy J. C., Dus K. M., Simpson E. R. and Waterman M. R.: Effect of adrenocorticotrophin on steroid 21-hydroxylase synthesis and activity in cultured bovine adrenocortical cells, increased synthesis in the absence of increased activity. *J. Biol. Chem.* 258 (1983) 9398–9405.
4. Simpson E. R. and Waterman M. R.: Steroid hormone biosynthesis in the adrenal cortex and its regulation by adrenocorticotropin. In *Endocrinology*. 2nd Ed. (Edited by L. J. DeGroot). WB Saunders, PA (1989) Vol. III, pp. 1543–1556.
5. Chang C-W., Naseeruddin S. A. and Hornsby P. J.: The response of 21-hydroxylase messenger ribonucleic acid levels to

adenosine 3′,5′-monophosphate and 12-*o*-tetradecanoylphorbol-13-acetate in bovine adrenocortical cells is dependent on culture conditions. *Endocrinology* **128** (1991) 604–610.
6. Provencher P. H., Tremblay Y., Fiet J. and Bélanger A.: Effect of ACTH on steroidogenic enzymes in guinea pig fasciculata-glomerulosa cells: changes in activity and mRNA levels. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 59–67.
7. Narasimhulu S.: Uncoupling of oxygen activation from hydroxylation in the steroid c-21 hydroxylase of bovine adrenocortical microsomes. *Arch. Biochem. Biophys.* **147** (1971) 384–390.
8. Hornsby P. J.: Regulation of cytochrome *P*-450-supported 11β-hydroxylation of deoxycortisol by steroids, oxygen, and antioxidants in adrenocortical cell cultures. *J. Biol. Chem.* **155** (1980) 4020–4027.
9. Hornsby P. J.: Regulation of 21-hydroxylase activity by steroids in cultured bovine adrenocortical cells: possible significance for adrenocortical androgen synthesis. *Endocrinology* **111** (1982) 1092–1101.
10. Perkins L. M., Hall P. F. and Payne A. H.: Testosterone-enhanced oxygen-mediated degradation of *P*-450-17α in mouse leydig cell cultures. *Endocrinology* **122** (1988) 2257–2264.
11. Hornsby P. J. and Vrivello J. F.: The role of lipid peroxidation and biological antioxidants in the function of the adrenal cortex. Part 2. *Molec. Cell Endocr.* **30** (1983) 123–147.
12. Provencher P. H., Tremblay Y. and Bélanger A.: Effects of C19 steroids on adrenal steroidogenic enzyme activities and their mRNA levels in guinea-pig fasciculata-glomerulosa cells in primary culture. *J. Endocr.* **132** (1992) 269–276.
13. Hornsby P. J.: Cytochrome *P*-450-pseudosubstrate interactions and the role of antioxidants in the adrenal cortex. *Endocrine Res.* **30** (1986) 469–474.
14. Provencher P. H., Bélanger A. and Fiet J.: Effects of RU 486 on adrenal steroidogenesis in the guinea pig. *J. Endocr.* **130** (1991) 71–78.
15. Black V. H., Mierlak T., Katz T., Mia P., Huima T. and McNamara N.: Isolated guinea pig adrenocortical cells *in vitro*: morphology and steroidogenesis in control and ACTH-treated cultures. *Am. J. Anat.* **165** (1982) 225–248.
16. Provencher P., Lorrain A., Bélanger A. and Fiet J.: Steroid biosynthesis by glomerulosa-fasciculata cells in primary culture of guinea pig adrenals. *J. Steroid Biochem.* **36** (1990) 589–596.
17. Chirgwin J. J., Przbyla A. E., MacDonald R. J. and Ritter W. J.: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18** (1979) 5294–5299.
18. Tremblay Y., Ringler G. E., Morel Y., Mohandas T. K., Labrie F., Strauss J. F. III and Miller W. L.: Regulation of the gene for estrogenic 17-ketosteroid reductase lying on chromosome 17cen-q25. *J. Biol. Chem.* **264** (1989) 20,458–20,462.
19. Thomas P. S.: Hybridization of denaturated RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natn. Acad. Sci. U.S.A.* **77** (1980) 5201–5205.
20. Matteson K. J., Phillips J. A. III, Miller W. L., Chung B., Orlando P. J., Frisch H., Ferrandez A. and Burr I. M.: *P*450XXI (steroid 21-hydroxylase) gene deletion are not found in family studies of congenital adrenal hyperplasia. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 5858–5862.
21. Clemens J. A., Matheson B. A., Wines D. R., Brady J. M., MacDonald R. J. and Funder J. W.: Androgen dependence of specific kallikrein gene family members expressed in rat prostate. *J. Biol. Chem.* **263** (1988) 16,132–16,137.
22. Chouinard M. L. and Fevold H. R.: ACTH-induced increases in rabbit adrenal immunoreactive *P*-450 17α and *P*-450 21. *Molec. Cell. Endocr.* **68** (1990) 29–34.
23. Slania S. M. and Fevold H. R.: The enzyme specificity of ACTH stimulation of rabbit adrenal microsomal 17α-hydroxylase activity. *J. Steroid Biochem.* **16** (1982) 193–199.
24. Fevold H. R. and Brown R. L.: The apparent lack of stimulation of rabbit adrenal 21-hydroxylase activity by ACTH. A preliminary note. *J. Steroid Biochem.* **9** (1978) 583–584.
25. Reid G. K., Kargman S., Vickers P. J., Mancini J. A., Léveillé C., Ethier D., Miller D. K., Gillard J. W., Dixon R. A. F. and Evans J. F.: Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. *J. Biol. Chem.* **265** (1990) 19,818–19,823.
26. Lin D., Black S. M., Nagahama Y. and Miller W. L.: Steroid 17α-hydroxylase and 17,20-lyase activities of *P*450c17: contribution of serine 106 and *P*450 reductase. *Endocrinology* **132** (1993) 2498–2506.