Transcriptional activity of the hamster *CYP11B2* promoter in NCI-H295 cells stimulated by angiotensin II, potassium, forskolin and bisindolylmaleimide

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ABSTRACT

We studied the regulation of the hamster *CYP11B2* gene in the NCI-H295 cell line, which is known to produce aldosterone in response to stimulation by angiotensin II (AII) and KCl. Ten deletion plasmids harboring the 5'-untranslated region of the *CYP11B2* gene were used for chloramphenicol acetyltransferase (CAT) assays. Transient transfections showed progressively increasing basal promoter activity by constructs beyond the TATA box, with a peak occurring with the -167 bp construct which contains putative Ad1, Ad2, Ad5 and the newly reported -143/-161 *cis*-element sequences. The promoter activity was lower with the construct containing the putative Ad3 *cis*-element and increased with longer constructs. This indicates the presence of both inhibitory and stimulatory *cis*-elements in this area of the gene. Expression of the reporter gene of all constructs was stimulated by AII and KCl, with the exception of the construct containing only the TATA box, which showed 6-fold and 10-fold increases occurring with the -167 bp deletion plasmid. The patterns of increase in CAT activity with AII and KCl treatment were similar, showing that these two regulators can stimulate hamster *CYP11B2* promoter activity through common *cis*-elements. The calcium channel antagonist nifedipine blocked the stimulatory effects of KCl on CAT activity, showing the involvement of calcium channels in the regulation of *CYP11B2* gene transcription by KCl. 12-*O*-Tetradecanoylphorbol 13-acetate, a known stimulator of the protein kinase C (PKC) signaling

pathway, was without significant effect on CAT activity. Bisindolylmaleimide, a specific inhibitor of PKC, had a significant enhancing effect (3.4- to 6-fold), indicating that PKC may negatively regulate the expression of the hamster *CYP11B2* gene in NCI-H295 cells. A mutation was induced in the sequence -143/-161 of the -350 bp construct in order to determine its importance in the regulation of hamster *CYP11B2* promoter activity. The stimulatory effects of AII, KCl, forskolin and bisindolylmaleimide on CAT activity were significantly less in the mutant than in the wild type. These results confirm that this *cis*-element is necessary in maintaining a high level of transcriptional activity in stimulated NCI-295H cells. In conclusion, using NCI-295H transfected cells, we have found that the 5'-untranslated region of the hamster *CYP11B2* gene possesses transcriptional activity with stimulatory and also inhibitory *cis*-elements; *CYP11B2* promoter activity can be stimulated by AII, KCl, forskolin, dibutyryl cAMP and bisindolylmaleimide. Our results suggest that this gene is positively regulated through the protein kinase A signaling pathway and through calcium channels, whereas PKC may have a negative regulatory effect upon the transcription of the *CYP11B2* gene. Furthermore, we have shown that the *cis*-element -143/-161 in the 5'-untranslated region of the hamster *CYP11B2* gene is important in maintaining a high level of promoter activity in stimulated NCI-295H cells.

Journal of Molecular Endocrinology (1998) **20**, 183-191

INTRODUCTION

Adrenal steroidogenesis, by which mineralocorticoids and glucocorticoids are produced,

involves numerous processes stemming from a common precursor, cholesterol (LeHoux *et al.* 1996a). The mineralocorticoid aldosterone is the product of three cytochrome P450 enzymes and

a 3 β -hydroxysteroid dehydrogenase (3 β -HSD), whereas the glucocorticoid cortisol is the product of four cytochrome P450 enzymes and 3 β -HSD. The final step in the synthesis of adrenal cortisol and aldosterone is mediated by two different enzymes in the adrenals of most animal species hitherto studied. The first, cytochrome P450 11 β -hydroxylase (P450C11), the product of the *CYP11B1* gene, is expressed in the zona fasciculata, yielding glucocorticoids. The second, cytochrome P450 aldosterone synthase (P450aldo), the product of the *CYP11B2* gene, is expressed in the zona glomerulosa, yielding aldosterone. P450aldo possesses a strong 11 β -hydroxylase activity and also 18-hydroxylase and 18-oxidase activities. This cytochrome mediates the 18-hydroxylation of corticosterone, yielding 18-hydroxycorticosterone, and then aldosterone (Miller 1988).

P450C11 and P450aldo are present in hamster (LeHoux *et al.* 1994, Véronneau *et al.* 1996), human (Mornet *et al.* 1989), mouse (Domalik *et al.* 1991) and rat (Imai *et al.* 1990, Nomura *et al.* 1993) adrenal cortex, whereas in the bovine (Morohashi *et al.* 1990) and porcine (Yanagibashi *et al.* 1986) glands, a single protein expressed in both the zona fasciculata and zona glomerulosa is responsible for the last steps of gluco- and mineralocorticoid formation.

Previous studies have shown that P450C11 and P450aldo are differently regulated (Tremblay *et al.* 1991, 1992, LeHoux & Tremblay 1992, Tremblay & LeHoux 1993). Adrenal glucocorticoid formation is mainly under the control of adrenocorticotropin (ACTH), whereas mineralocorticoid formation is primarily regulated by the renin-angiotensin system and potassium ions via the opening of calcium channels (LeHoux *et al.* 1996a). It has been shown that low-sodium and high-potassium diets can promote increases in P450aldo mRNA content and expression of its protein (Tremblay *et al.* 1991, 1992, LeHoux *et al.* 1996a,b). Low-sodium diets increase circulating angiotensin II (AII) levels via the renin-angiotensin system (Tremblay & LeHoux 1992).

A *CYP11B2* gene encoding P450aldo was isolated from a hamster genomic library (Coulombe *et al.* 1996, 1997). When compared with the gene of other species, the 5'-untranslated region (UTR) of the hamster *CYP11B2* gene possesses conserved sequences similar to Ad1, Ad2, Ad3, Ad4 (also named SF-1) and Ad5 (Honda *et al.* 1990, Morohashi & Omura 1990, Lala *et al.* 1992). Furthermore, footprinting analysis demonstrated an additional conserved sequence located in the hamster *CYP11B2* gene between -143 and -161 bp (-143/-161) (Coulombe *et al.* 1996, 1997).

Using chloramphenicol acetyltransferase (CAT) assays, we previously studied the regulatory activity

of the 5'-UTR of the hamster *CYP11B2* gene in Y1 cells (Coulombe *et al.* 1996, 1997). These experiments demonstrated that the hamster *CYP11B2* promoter was responsive to forskolin, indicating that the gene is controlled by the protein kinase A signaling pathway. We also reported that the *cis*-element -143/-161 played a negative regulatory role in *CYP11B2* gene transcription in Y1 cells. However, this cell line did not respond to stimulation by AII or potassium ions by controlling *CYP11B2* promoter activity (Coulombe *et al.* 1996, 1997).

In the present study, we describe the regulation of hamster *CYP11B2* promoter transcriptional activity in the NCI-H295 cell line. This cell line (Gazdar *et al.* 1990) was chosen because it expresses the three main pathways of adrenal steroidogenesis, namely mineralocorticoid, glucocorticoid and C₁₉ steroid production, and because, in contrast with the Y1 line, it is responsive to potassium and AII in addition to forskolin (Bird *et al.* 1995). We found that hamster *CYP11B2* promoter activity is stimulated by AII, potassium ions, forskolin, dibutyryl (dBt) cAMP and bisindolylmaleimide; furthermore, in NCI-H295 cells, the *cis*-element -143/-161 enhances transcriptional activity of the *CYP11B2* promoter.

MATERIALS AND METHODS

Deletion plasmids

The pCAT basic vector (Promega, Madison, WI, USA) was modified by removing AP-1 and AP-1-like *cis*-elements and adding two polyadenylation signal sequences upstream of the multicloning site (Coulombe *et al.* 1997). This modified vector was used to construct ten *CYP11B2* gene deletion plasmids. These deletion plasmids were constructed to study putative Ad1, Ad2, Ad5 Ad3 and Ad4 *cis*-elements of the gene. As shown in Fig. 1, the shortest deletion plasmid -63 bp contained the TATA box only; the -83 bp contained Ad1; the -102 bp contained Ad2; the -134 bp contained Ad5; the -167 bp contained the recently reported new *cis*-element -143/-161 (Coulombe *et al.* 1997); the -328 bp contained Ad3; the -350 bp contained Ad4; the three other deletion plasmids contained respectively the first -486 bp, -2458 bp, and -3722 bp of the 5'-UTR of the gene.

Mutation of the -143/-161 *cis*-element

The sequence 5'-ggtaagacacttctactga-3' was changed to 5'-aagacatggtaccatagct-3' which comprised a KpnI site (underlined). To do so, PCRs were

FIGURE 1. Schematic representation of the 5'-UTR of the hamster *CYP11B2* gene. A series of deletion constructs containing progressively shorter fragments of the promoter region were prepared. These deletion plasmids were constructed in order to study putative Ad1, Ad2, Ad5, Ad3, and Ad4 *cis*-elements of the gene. The shortest deletion plasmid - 63 bp contained the TATA box only; the - 83 bp contained Ad1; the - 102 bp contained Ad2; the - 134 bp contained Ad5; the - 167 bp contained the recently reported *cis*-element - 143/- 161; the - 328 bp contained Ad3; the - 350 bp contained Ad4; the three other deletion plasmids contained respectively the first - 486 bp, - 2458 bp, and - 3722 bp of the 5'-UTR of the gene.

performed on the - 350 construct (containing the putative Ad4 *cis*-element) using two sets of primers; first: 5'-catgcatgcagaaaccaaggtcttcta-3' (sense) with an SphI site and 5'-catggtaccatgtcttagctccctaataccatccctc-3' (antisense) with a KpnI site; second: 5'-atcggtaccatagctcaggtgagcccgactatg-3' (sense) with a KpnI site and 5'-catctgcagccctccctactctg tcg-3' (antisense) with a PstI site. The fragments were then digested with the corresponding enzymes and ligated with the pCAT vector digested with SphI and PstI.

Cell culture

NCI-H295 cells were maintained in Dulbecco's modified Eagle's medium/Ham F12 (1:1) supplemented with 1% ITS Premix (Becton Dickinson Labware, Bedford, MA, USA), 2% UstroSer SF (Bio Septra SA, Villeneuve la Garenne, France), 200 µg/ml streptomycin and 200 U/ml penicillin G. Cells were grown at 37 °C under 5% CO₂.

Transient transfections and CAT assays

A total of 5×10^5 cells were seeded on to six-well plates and cultured until 80-90% confluent. The cells were transiently transfected for 16 h with plasmid constructs and β-galactosidase plasmid using Lipofectamine, according to the manufacturer's protocol (Gibco-BRL, Burlington, Ontario, Canada). The medium was then changed for one containing different regulators (25 µM forskolin; 16 mM KCl; 10^{-7} M AII; 100 nM staurosporine; 200 nM bisindolylmaleimide; 16.2 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA); 1 mM dBtAMP; 1 µM BAYK 8644; 10 µM nifedipine) and cells were cultured for another 48 or 60 h. The same concentrations of stimuli/inhibitors were used in experiments in which

aldosterone production and CAT activity were determined. Cell lysates were prepared by freeze-thawing, and CAT activity was assayed by silica gel thin layer chromatography using [¹⁴C]chloramphenicol (Gorman *et al.* 1982); radioactivity was quantified using an optical imager (PhosphorImager SF, Molecular Dynamics, Sunnyvale, CA, USA). CAT activity was calculated as the percentage of chloramphenicol converted to acetylated forms and corrected for transfection efficiency with the corresponding β-galactosidase activity (β-Gal ELISA; Boehringer-Mannheim GmbH, Mannheim, Germany). All experiments were performed in triplicate and repeated at least three times. Statistical significance was determined using a Mann-Whitney U test, and a value of $P < 0.05$ was considered significant.

Steroid quantification

The level of aldosterone in incubation media was measured by the Coat-A-Count RIA procedure (Diagnostic Products, Los Angeles, CA, USA).

RESULTS

Effects of regulators on aldosterone output of NCI-H295 cells

Significant increases over the basal level of aldosterone were produced (mean ± S.E.M., $n=3$, $P < 0.001$) by forskolin ($360 \pm 24\%$), dBtAMP ($550 \pm 9.8\%$), AII ($430 \pm 21.3\%$), bisindolylmaleimide ($300 \pm 4.1\%$), KCl ($430 \pm 13.9\%$) and BAYK 8644 ($520 \pm 15.5\%$), and decreases by staurosporine ($63 \pm 7.8\%$) and TPA ($25 \pm 3.9\%$). Nifedipine had no effect on the basal level of aldosterone but it

completely prevented the increases produced by both BAYK 8644 and KCl.

Effects of regulators on the expression of CAT activity in NCI-H295 cells

In a first series of experiments, we investigated the effects of the above mentioned regulators on the -486 bp construct. This -486 bp region of the 5'-UTR of *CYP11B2* gene showed promoter activity that was stimulated by dBtcAMP and forskolin (720 and 300% respectively) (Fig. 2B). AII also stimulated CAT activity (450%) (Fig. 2A), whereas TPA, a known stimulator of the protein kinase C (PKC) signaling pathway, had no significant effect. However, with a specific inhibitor of the PKC signaling pathway, namely bisindolylmaleimide, a 340% increase in CAT activity was obtained. Staurosporine, a broad spectrum inhibitor of kinases, reduced CAT activity to one third of control value.

KCl stimulated CAT activity by 700% (Fig. 2C) while the calcium channel activator BAYK 8644 showed an increase of 820%. The calcium channel antagonist nifedipine had no effect on CAT activity but prevented the increases produced by both BAYK 8644 and KCl, demonstrating the involvement of calcium channels in the mode of action of KCl on the transcription of *CYP11B2*.

Expression of basal CAT activity

Ten deletion plasmids were used to study the promoter regulatory regions of the hamster *CYP11B2* gene. As shown in Fig. 3, basic and TATA box plasmids showed only a small background CAT activity. Compared with the basic plasmid containing no *CYP11B2* insert, the promoter activity gradually and significantly ($P < 0.05$) increased with the augmentation of the length of deletion plasmids containing respectively the putative *cis*-elements Ad1, Ad2 and Ad5; a peak of activity was reached with the -167 bp construct, containing the $-143/-161$ *cis*-element. A significant ($P = 0.001$) decrease in CAT activity occurred thereafter with the -328 pb construct containing Ad3, to increase again with longer constructs.

Effects of AII and KCl on the expression of CAT activity by the ten deletion plasmids

As shown in Fig. 4, AII (middle panel) and KCl (lower panel) significantly ($P < 0.05$) stimulated the expression of the reporter gene of all constructs except those of basic and TATA box. A peak of activity (600% with AII; 1000% with KCl) occurred

FIGURE 2. Effects of various regulators on CAT gene expression upon transfection of NCI-H295 cells with the -486 bp construct. At 16 h after transfection, the medium was changed for one containing different regulators. (A) Control (C); 10^{-7} M angiotensin II (AII); 16.2 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA); 100 nM staurosporine (Stauro); 200 nM bisindolylmaleimide (Bis). (B) Control (C); 25 μ M forskolin (Fors); 1 mM dBtcAMP (cAMP). (C) Control (C); 16 mM KCl; 1 mM BAYK 8644 (Bk); 10 μ M nifedipine (Nf), and cells were cultured for another 48 or 60 h. Results shown are representative of three experiments performed in triplicate (means $\pm$ S.E.M.). * $P < 0.05$, experimental vs control. NS, not significant.

FIGURE 3. Basal transcriptional activities of truncated hamster *CYP11B2* gene promoters. NCI-H295 cells were transfected with the basic plasmid containing no *CYP11B2* insert and with various deletion plasmids, and cultured for 48 h. Transfection efficiency was standardized by β -galactosidase analysis as described in Materials and Methods. Results shown are from one experiment representative of three experiments performed in triplicate (means $\pm$ S.E.M.). * $P < 0.05$, compared with the basic plasmid containing no *CYP11B2* insert. NS, not significant.

when the -167 bp deletion plasmid which contains the $-143/-161$ *cis*-element was used. Compared with the -167 bp construct, the relative increase was significantly ($P=0.02$) smaller with the deletion plasmid containing the putative Ad3 *cis*-element; with longer constructs, the percentage increase returned towards that of the -167 bp construct.

Mutation in the $-143/-161$ *cis*-element of the hamster *CYP11B2* gene

As mentioned above, a peak of basal CAT activity and also AII- and KCl-stimulated activity occurred when NCI-H295 cells were transfected with the -167 bp deletion plasmid which contains the $-143/-161$ *cis*-element. This region was thus mutated in the Ad4 construct to study its implication in the *CYP11B2* gene regulation. Fig. 5 shows that mutation of the $-143/-161$ *cis*-element decreased basal CAT activity, although not significantly. However, the stimulating effects of all regulators on the mutant CAT activity were significantly lower than those of the wild type. These results thus confirm that the $-143/-161$ *cis*-element is necessary to maintain a high level of *CYP11B2* transcriptional activity in NCI-H295 cells.

DISCUSSION

In this work we report that the 5'-UTR of the hamster *CYP11B2* gene possesses promoter activity that can be stimulated by AII, KCl, forskolin, dBtcAMP and bisindolylmaleimide. These results

FIGURE 4. Basal, AII- and KCl-stimulated transcriptional activities of truncated hamster *CYP11B2* gene promoters. NCI-H295 cells were transfected with the basic plasmid containing no *CYP11B2* insert and with various deletion plasmids and cultured for 48 h without (basal promoter activity, upper panel) or with 10^{-7} M AII (middle panel) or 16 mM KCl (lower panel). Transfection efficiency was standardized by β -galactosidase analysis as described in Materials and Methods. Results shown are from one experiment representative of three experiments performed in triplicate (means $\pm$ S.E.M.). * $P < 0.05$; comparisons were made between deletion plasmids and the basic plasmid containing no *CYP11B2* insert. Comparisons were also made for each plasmid between stimulated and non-stimulated conditions. NS, not significant.

were obtained using the CAT assay carried out on NCI-H295 cells that were transfected with *CYP11B2* deletion plasmids. These deletion plasmids were constructed to study the effects of conserved sequences among the rat, mouse, human

FIGURE 5. Mutation of the $-143/-161$ *cis*-element in the Ad4 construct. The sequence 5'-ggtaagacacttactga-3' was changed to 5'-aagacatgggtaccatagct-3'. Basal as well as AII-, KCl-, bisindolylmaleimide- and forskolin-stimulated CAT activities were determined. When compared with the wild type (-350), all CAT activities were diminished with the mutant (mut-350). Results shown are the mean $\pm$ S.E.M. from one experiment performed in triplicate using three different plasmid preparations, and representative of three different experiments. * $P < 0.05$; in the four conditions studied, the stimulated CAT activity of the mut-350 was significantly lower than that of the wild type -350.

and hamster *CYP11B2* genes, namely Ad1, Ad2, Ad5, Ad3 and Ad4 (Honda *et al.* 1990, Morohashi & Omura 1990, Lala *et al.* 1992), and a newly reported conserved sequence between -143 bp and -161 bp of the 5'-UTR of the hamster gene (Coulombe *et al.* 1996, 1997). In NCI-H295 cells, a peak in basal transcription occurred with the -167 bp construct which contains many *cis*-elements, namely Ad1, a known cAMP *cis*-element in the mouse *cyp11b2* gene (Mouw *et al.* 1989), Ad2, Ad5 and the $-143/-161$ *cis*-element. However, when Y1 cells were transfected with the same hamster *CYP11B2* deletion plasmids, it was not the -167 bp construct that showed the highest transcriptional activity; on the contrary, its activity was lower than that of the -134 construct, suggesting an inhibitory function for the $-143/-161$ bp *cis*-element in Y1 cells (Coulombe *et al.* 1996, 1997). These opposing results indicate the possibility that interactions between the $-143/-$

-161 *cis*-element and other binding protein(s) may differ between NCI-H295 and Y1 cells. Indeed, positive or negative regulation in this region of the gene could well depend upon the presence, the absence, or a difference in concentration of specific binding protein(s) in these two cell types. In contrast with NCI-H295 cells, Y1 cells do not produce aldosterone because they lack certain steroidogenic enzymes. This strongly suggests the possibility of deficient expression of important transcription factor(s) involved in the expression of steroidogenic genes in Y1 cells. The presence of a putative inhibitory sequence between the Ad5 and Ad4 *cis*-elements of the mouse *cyp11b2* gene has also been reported in transfection studies using Y1 cells (Bogerd *et al.* 1990). This inhibitory sequence on the mouse *cyp11b2* gene corresponds to a sequence of the hamster *CYP11B2* promoter located upstream of the $-143/-161$ *cis*-element but overlapping it by

five nucleotides. In contrast, no such inhibitory *cis*-elements have been found in the first –2015 bp of the 5'-UTR of the human *CYP11B2* gene (Clyne *et al.* 1997).

Forskolin and cAMP stimulate the transcriptional activity of hamster *CYP11B2* deletion plasmids in NCI-H295 cells, demonstrating participation of the protein kinase A signaling pathway in modulating the expression of this gene. This is in agreement with results obtained using Y1 cells (Coulombe *et al.* 1996, 1997).

Under the experimental conditions used, an addition of 10^{-7} M ACTH to the incubation medium of NCI-H295 cell culture did not induce significant increases in either aldosterone or cortisol production; this is why we did not study the effect of ACTH on hamster *CYP11B2* promoter activity. Our results are in agreement with those published by Clyne *et al.* (1996), who found that ACTH provoked an increase in the human *CYP11B2* promoter activity in transfected Y1 but not NCI-H295 cells. The lack of response of NCI-H295 cells may tentatively be attributed to the low level of ACTH receptor expression in these cells (Rainey *et al.* 1993, Mountjoy *et al.* 1994), or to an unknown defect located between ACTH receptor and adenylate cyclase, since, as shown in this report, dBtcAMP and also forskolin were able to induce the hamster *CYP11B2* promoter activity.

In contrast with Y1 cells (Coulombe *et al.* 1997), in NCI-H295 cells, AII and KCl were able to stimulate the promoter activity of the hamster *CYP11B2* gene. The concentration 10^{-7} M AII was chosen because it has been shown to maximally stimulate aldosterone production in NCI-H295 cells (Bird *et al.* 1993). The reported stimulatory effects of AII on the transcriptional activity of the mouse (Holland *et al.* 1995) and human (Clyne *et al.* 1996) *CYP11B2* promoter in NCI-H295 cells are in agreement with our results. More recently, the same group (Clyne *et al.* 1997) identified two *cis*-elements in the human *CYP11B2* promoter that are required for full basal reporter gene activity and for maximal induction by either cAMP or calcium signaling pathways. One of these *cis*-elements resembles a consensus cAMP response element; the other, located in the Ad5 area, demonstrated binding of the orphan nuclear receptors SF-1 and chicken ovalbumin upstream promoter transcription factor (COUP-TF), as determined by the absence of binding in the presence of antibodies directed against SF-1 and COUP-TF. In human *CYP11B2* gene, the last seven nucleotides TGACCTT in the Ad5 area involved in the binding of SF-1 forms an SF-1-like sequence. A similar sequence is not present in the Ad5 area of the hamster, rat and

mouse *CYP11B2* genes, and therefore SF-1 is unlikely to contribute to the enhancing effect observed with the hamster *CYP11B2* –134 bp deletion plasmid containing the putative Ad5 *cis*-element. In our study, maximal AII or KCl stimulation occurred with the –167 bp transfectant which contains the previously mentioned –143/–161-protected *cis*-element. This *cis*-element was mutated to further evaluate its importance to *CYP11B2* promoter activity. Mutations were effected on the –350 bp deletion plasmid containing Ad4 in order to allow interactions with other putative regulatory factors that could intervene beyond the mutated region. The basal CAT activity of the mutant fell but was not significantly decreased compared with that of the wild type. However, stimulation by KCl, AII, forskolin and bisindolylmaleimide was significantly less with the mutant, indicating that this *cis*-element is important in maintaining a full response of transcriptional activity to these regulators.

Our results showing that nifedipine inhibited the stimulatory effect of KCl on the transcriptional activity of *CYP11B2* and on aldosterone output are in agreement with the report of Bird *et al.* (1995), who showed that this calcium channel antagonist blocked the KCl-induced aldosterone output in NCI-H295 cells. Potassium acts by increasing intracellular calcium through the opening of nifedipine-sensitive voltage-dependent calcium channels of the plasma membrane (Rainey *et al.* 1993). Taken together these results indicate that the entry of calcium plays an important role in the stimulatory action of KCl on aldosterone synthase promoter activity and aldosterone synthesis in NCI-H295 cells. Moreover, Pezzi *et al.* (1997) have reported that treatment with either calmidazolium, a calmodulin inhibitor, or KN93, an inhibitor of calmodulin-dependent kinases, completely inhibited potassium-stimulated expression of P450aldo mRNA with little effect upon AII- or dBtcAMP-stimulated induction of the transcript. This suggests that potassium, cAMP and AII effects on transcriptional activity may be secondary to differences in signal-transduction mechanisms.

In this study we used 16 mM KCl to stimulate NCI-H295 cells since this dose was reported to give maximal aldosterone production (Rainey *et al.* 1994). Also, Bird *et al.* (1995) showed a dose-dependent increasing effect of potassium up to 16 mM on P450C17 mRNA but not on that of 3 β -HSD mRNA, demonstrating the specificity of the stimulus towards specific genes and indicating that these effects are of physiological significance.

The fact that bisindolylmaleimide, a potent and selective inhibitor of PKC (Toullec *et al.* 1991),

stimulated both the transcriptional activity of hamster *CYP11B2* deletion plasmids and aldosterone output of transfected NCI-H295 cells suggests that the PKC pathway plays a negative regulatory role in aldosterone synthesis. Furthermore, and in agreement with this interpretation, prolonged incubation with TPA, the activator of PKC, did not increase *CYP11B2* transcriptional activity. It has also been reported (Clyne *et al.* 1996) that TPA has no effect on the activity of human *CYP11B2* promoter in NCI-H295 cells. Thus the chronic stimulatory action of AII on transcription of hamster *CYP11B2* appears not to be mediated through a PKC pathway. The effect of shorter incubation times with TPA on hamster *CYP11B2* promoter activity was not studied in this work. However, Bird *et al.* (1993) reported that short-term treatment of NCI-H295 cells with AII resulted in a 450% increase in total inositol phosphates and in a significant increase in aldosterone output, indicating that the acute effect of AII on aldosterone output is mediated by inositol phosphates.

Staurosporine lowered *CYP11B2* transcriptional activity and aldosterone output in NCI-H295 cells, but had a stimulatory effect on the transcriptional activity of the *CYP11B2* gene and on steroid output in Y1 cells (Reyland 1993, Coulombe *et al.* 1997). Staurosporine, although a potent inhibitor of PKC, is poorly selective when assayed against other protein kinases (Toullec *et al.* 1991), and discrepancies between the response of the two cell types to this product can also be tentatively explained by differences in the signaling pathways between NCI-H295 and Y1 cells, the latter being deficient, as previously mentioned, in expressing certain steroidogenic genes involved in aldosterone, cortisol and dehydroepiandrosterone synthesis. Also, the difference observed between the effects of staurosporine and bisindolylmaleimide might be due to the presence in NCI-H295 cells of PKC isoenzymes which may be affected differently by various inhibitors and activators.

In conclusion, we found that the 5'-UTR of the hamster *CYP11B2* gene in transfected NCI-H295 cells possesses transcriptional activity that can be stimulated by AII, KCl, forskolin, dBtcAMP and bisindolylmaleimide. Our results suggest that this gene is regulated, at least in part, through the protein kinase A signaling pathway and through calcium channels, whereas PKC might negatively regulate the transcription of the *CYP11B2* gene. We have shown that the *cis*-element -143/-161 in the 5'-UTR of the hamster *CYP11B2* gene is important in maintaining high promoter activity in NCI-295H cells.

ACKNOWLEDGEMENTS

We are grateful to Dr D Shapcott for invaluable advice and to Dr WE Rainey for providing NCI-H295 cells. This work was supported by a grant from the Medical Research Council of Canada (MT10983), and the Heart and Stroke Foundation of Canada.

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RECEIVED 10 July 1997