

Expression of genes encoding steroidogenic enzymes in the bovine corpus luteum

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Summary. To examine the regulation of P-450_{SCC} expression at the molecular level, a transfection protocol specific for bovine luteal cell cultures was developed. Among several commonly used transfection methods, electroporation yielded highest transfection efficiencies. Transfection of primary cultures of bovine luteal cells with chimaeric DNA constructs containing increasing deletions of the 5'-flanking region of P-450_{SCC} fused to the chloramphenicol acetyl transferase (CAT) reporter gene allowed two conclusions. Firstly, sequences of the P-450_{SCC} 5'-flanking region were capable of conferring basal expression and cAMP responsiveness to the CAT reporter gene and secondly, the region -186 to -100 bp appears to be required for these two types of regulation of gene expression. On the other hand, 5'-flanking regions of P-450_{17 α} were not capable of conferring any regulation of gene expression to the CAT reporter gene in these cells. Thus, the physiologically observed regulation of the endogenous cytochromes P-450_{SCC} and P-450_{17 α} is closely reflected by CAT reporter gene expression. These experiments will allow investigation of the molecular mechanisms underlying the regulation of the genes encoding steroidogenic enzymes throughout the ovarian cycle.

Keywords: gene expression; corpus luteum, cytochrome P-450; transfection; luteal cell cultures

Introduction

Steroid hormones are essential in regulating and co-ordinating normal development, differentiation and function of higher organisms. The biosynthesis of these hormones is the result of a complex sequence of chemical modifications of the common precursor cholesterol and involves specific enzymes, many of which belong to the superfamily of cytochromes P-450. These enzymes act as mixed-function oxidases and require molecular oxygen as well as reducing equivalents to metabolize their substrates (Ortiz de Montellano, 1986).

In the ovary, three distinct cytochromes P-450 (side chain cleavage cytochrome P-450 (P-450_{SCC}); 17 α -hydroxylase cytochrome P-450 (P-450_{17 α}) and aromatase cytochrome P-450 (P-450_{AROM})) act in concert to allow for the episodic pattern of steroid hormone production. Throughout the ovarian cycle, follicles mature under the influence of FSH and LH, producing increasing amounts of oestrogens. Triggered by the LH surge, follicular thecal and granulosa cells both differentiate into luteal cells, now producing large amounts of steroid hormones, principally progesterone. The massive production of progesterone in the luteal phase of the bovine ovarian cycle is accompanied by increased P-450_{SCC} protein and mRNA levels (Hansel & Echterkamp, 1972; Rodgers *et al.*, 1986, 1987), and a corresponding decline in expression of P-450_{17 α} . The stimulatory effect of gonadotrophins is thought to be mediated, at least in part, by cAMP (Marsh, 1976). However, an increasing number of studies (Hansel *et al.*, 1987; Davis *et al.*, 1989; Wiltbank *et al.*, 1989; Benhaim *et al.*, 1990) demonstrate that the luteotrophic effect of LH also may include other second messenger systems, such as phospholipase C acting via inositol trisphosphate/calcium

(IP₃/Ca) and diacyl glycerol/protein kinase C (DAG/PKC). In addition, several growth factors, including insulin-like growth factor 1 (IGF-1) and insulin as well as angiotensin II, have been reported to influence expression of P-450_{SCC} (Veldhuis *et al.*, 1986; Stirling *et al.*, 1990). The elucidation of the molecular mechanisms underlying the regulation of expression of the genes encoding steroidogenic cytochromes P-450 is therefore of great importance towards a better understanding of the physiological functions of the ovary.

The following study was undertaken to determine whether primary cultures of bovine luteal cells provide a suitable system for the study of regulation of expression of the cytochrome P-450_{SCC} gene. As a first step, it was necessary to establish a transfection protocol to introduce chimaeric DNA constructs efficiently into the luteal cell cultures. Secondly, these chimaeric DNA constructs were transfected into primary cultures of bovine luteal cells and the functional properties of the specific P-450_{SCC} and P-450_{17 α} 5'-flanking sequences were determined.

Materials and Methods

Cell cultures. Bovine corpora lutea (CL) were obtained from a local slaughterhouse and cell cultures were prepared as described by Stirling *et al.* (1990). As required, the corpora lutea were staged according to the criteria of Ireland *et al.* (1978). The luteal cell cultures were grown in McCoy's 5A medium supplemented with selenium (1 ng/ml), transferrin (1 μ g/ml) and 2.5% fetal calf serum (FCS). Activation of the cAMP-dependent second messenger system was achieved by addition of forskolin (25 μ M), luteinizing hormone (LH; 10 ng/ml) and dibutyryl cAMP (Bt₂cAMP; 1 mM).

Immunocytochemistry. To evaluate the localization and amount of cytochrome P-450_{SCC} protein in bovine luteal cell cultures, immunocytochemistry was applied to cells grown on chamber slides. The procedure used for the immunostaining included; fixation with paraformaldehyde (3%), permeabilization with Triton X-100 (1%), incubation with the primary antibody (1/600 dilution of a polyclonal IgG preparation raised in a rabbit against purified bovine P-450_{SCC}) in a humidified chamber at room temperature for 1 h and visualization of specific binding by using a fluorescein-coupled secondary antibody. To determine the degree of non-specific binding, preimmune serum was used in the first incubation.

mRNA isolation and Northern blot analysis. Total mRNA was prepared according to the procedure of Chomczynski & Sacchi (1987). Concentrations of the mRNA preparations were determined by measuring the respective absorption at 260 nm. Total mRNA (10 μ g) was loaded onto a formaldehyde-agarose gel and following electrophoresis, electrotransferred to a nylon membrane (zeta probe (Bio-Rad, Richmond, CA, USA)) and hybridized with labelled cDNA specific for P-450_{SCC} as described by Doody *et al.* (1990).

Transfection of cell cultures and CAT assay. The evaluation of a suitable transfection protocol was carried out employing commonly used transfection methods such as calcium-phosphate (Fordis & Howard, 1987), DEAE-Dextran (Zuber *et al.*, 1986) and electroporation (Neumann *et al.*, 1982). Electroporation was carried out using the Cell Porator[®] from Bethesda Research Laboratories (BRL, Bethesda, MD, USA). Highest transfection efficiency was observed when cells were discharged twice at room temperature in McCoy's 5A medium containing no serum. The electrical discharge delivered a peak voltage of 750 V/cm at 1180 μ F (the capacitance setting determines the fall time of the voltage) to the cell suspension. After electroporation, cells were diluted in McCoy's 5A containing 2.5% FCS and plated in 10 cm dishes. Treatment with or without forskolin (25 μ M) was started immediately. CAT-reporter gene (chloramphenicol acetyltransferase) activity was determined according to the method of Fordis & Howard (1987). For each CAT assay, 100 μ g of cytosolic protein extract were used. Radioactivity was visualized by autoradiography on thin-layer chromatography plates and quantitation was achieved by scintillation counting.

DNA reporter gene constructs. The cloning and characterization of several chimaeric DNA constructs, containing increasing deletions of the 5' regulatory regions of P-450_{SCC} and P-450_{17 α} upstream of the CAT reporter gene have been described previously (Ahlgren *et al.*, 1990; Lund *et al.*, 1990).

Results

Characterization of primary bovine luteal cell cultures

Previous studies have indicated that primary bovine luteal cell cultures maintain their responsiveness to LH. After 24 h of LH stimulation, progesterone concentrations were increased 15-fold over control and mRNA levels specific for P-450_{SCC} were elevated about 3–5-fold (Stirling *et al.*,

1990). To evaluate the most efficient way to stimulate the cAMP-dependent second messenger system, LH, Bt_2cAMP and forskolin were compared to their effect on progesterone accumulation. Forskolin at a concentration of $25 \mu M$ appeared to be the best stimulator (data not shown). As shown in a time-course experiment (Fig. 1) the stimulatory effect of forskolin is already detectable after 3 h and progesterone formation was maximally increased between 12 and 24 h (10-fold). Moreover, the stimulatory effect of forskolin persisted for up to 48 h. Similar regulation of progesterone accumulation, albeit at a lower level, could be observed in luteal cells kept for up to 2 weeks in culture and in first-passage luteal cells.

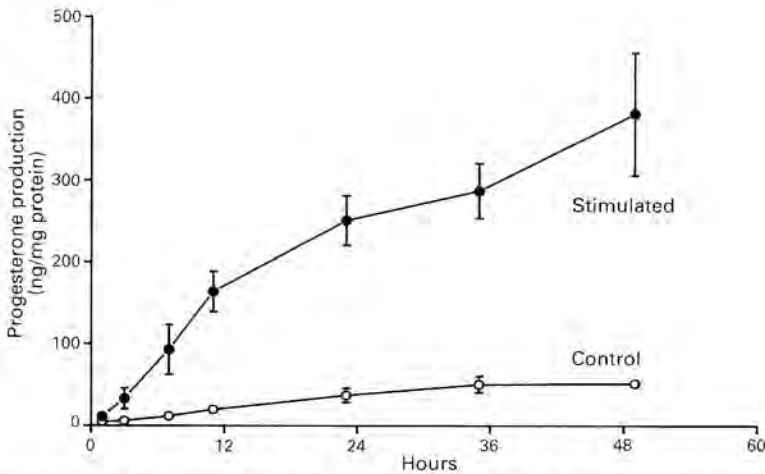


Fig. 1. Time course of progesterone production in primary cultures of bovine luteal cells with (●) or without (○) the addition of forskolin. Progesterone was measured by RIA and the values represent the mean \pm s.e.m. from triplicate dishes.

To determine the homogeneity of the luteal cell cultures with respect to P-450_{SCC} protein content and distribution, immunocytochemistry experiments were carried out. Incubation with an antibody specific for bovine P-450_{SCC} yielded positive staining for almost all of the cells (Fig. 2a). This indicated that the large and small luteal cells do express P-450_{SCC} equally and further provided evidence that no detectable non-steroidogenic cells, such as fibroblasts or endothelial cells, contaminate the luteal cell cultures. The complete absence of fluorescence in the preimmune serum-treated cell cultures (Fig. 2b) supported the specificity of the P-450_{SCC} antibody.

Northern blot analysis of luteal cell cultures using a cDNA probe specific for bovine cytochrome P-450_{SCC} demonstrated that forskolin stimulation (24 h) increased P-450_{SCC} mRNA levels (Fig. 3). Cell cultures derived from early and late stages of corpora lutea responded in a similar fashion to the treatment.

Transfection of primary cultures of bovine luteal cells

To study regulation of gene expression by using chimaeric DNA reporter gene constructs, a transfection protocol for the particular cell type in question has to be developed. For this purpose some commonly used transfection methods, such as calcium-phosphate, DEAE-Dextran and electroporation were examined for their potential to introduce chimaeric DNA constructs into the primary cultures of bovine luteal cells. The calcium-phosphate and DEAE-Dextran transfection methods both led to massive cell death and undetectable reported gene activity. However, transfection by means of electroporation consistently resulted in high transfection efficiencies as evidenced

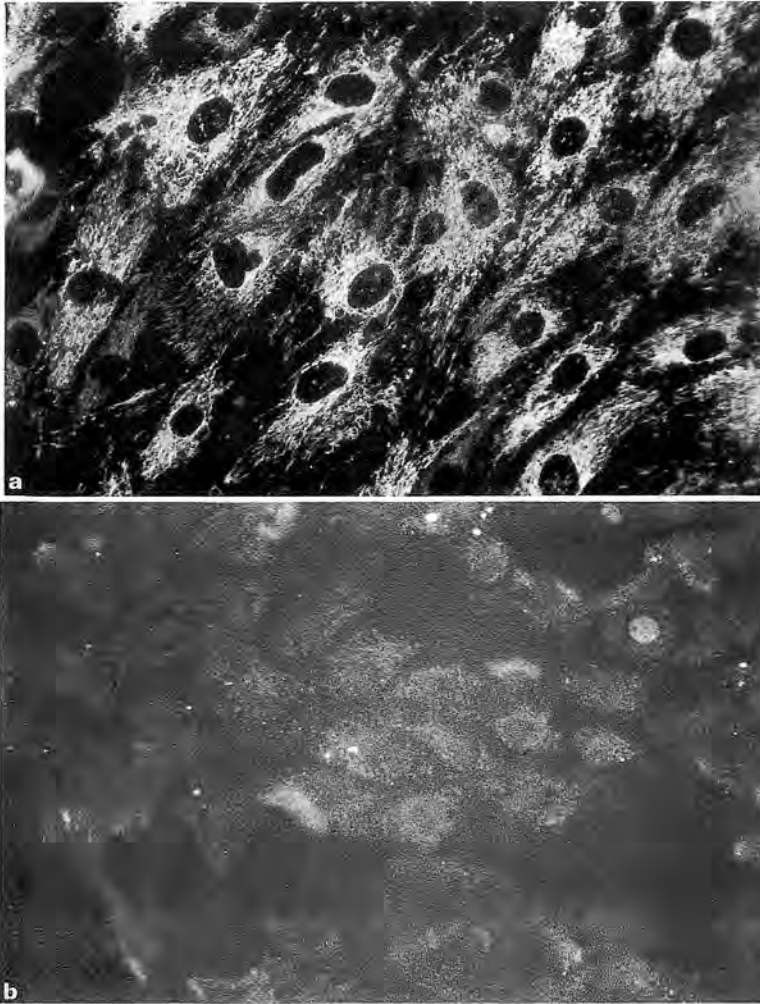


Fig. 2. Primary cultures of bovine luteal cells immunostained with (a) a specific P-450_{SCC} antibody and (b) pre-immune serum. Experimental conditions are given in 'Materials and Methods'.

by high levels of CAT activity. Optimal DNA uptake was achieved using two consecutive electrical discharges (750 V/cm, 1180 μ F). Higher peak voltage of the electrical discharge increased transfection efficiency further but at the same time dramatically increased cell death, resulting in a decreased net CAT activity (data not shown). To characterize the electroporation protocol further, the effect of the incubation time after transfection, as well as the influence of the total amount of DNA used per experiment on the relative CAT activity were evaluated. As shown in Fig. 4(a), the amount of DNA used per transfection had a pronounced effect on the respective CAT-activity levels: 2.5 μ g and 5 μ g of the RSV-CAT DNA construct yielded modest levels of CAT activity, whereas 10 μ g and 20 μ g of DNA resulted in 16% and 47% conversion, respectively. In an additional experiment it was determined that 100 μ g of the specific P-450_{SCC} CAT constructs are required for CAT-activity to fall within the linear range of the assay (data not shown). A time-course experiment using 20 μ g RSV-CAT DNA revealed that a significant amount of CAT activity was detectable within 12 h after transfection (Fig. 4b). The increase of CAT activity after 24, 36 and



Fig. 3. Northern blot analysis of primary cultures of bovine luteal cells prepared from early and late stage corpora lutea treated for 24 h with (+) or without (-) forskolin. A radiolabelled P-450_{SCC} cDNA probe was used to detect specific mRNA levels: 10 µg total mRNA were loaded in each lane. Total mRNA from primary cultures of bovine adrenocortical cells (A) and total mRNA from primary cultures of ovarian connective tissue (C) were used as positive and negative controls, respectively.

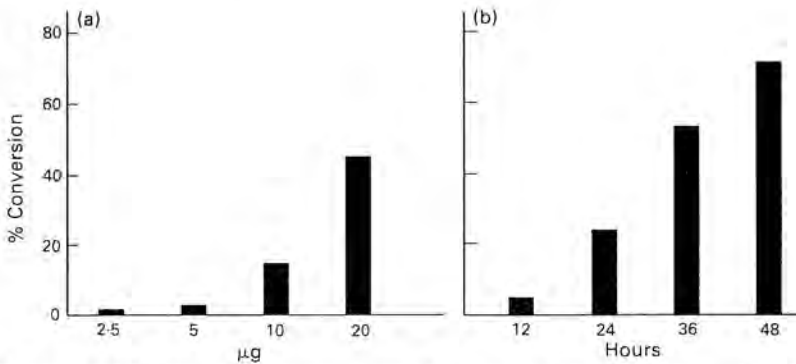


Fig. 4. Relative CAT activity (% conversion) of primary cultures of bovine luteal cells (a) transfected with different amounts of the RSV-CAT reporter gene construct or (b) incubated after transfection with RSV-CAT (20 µg) for different periods of time. Details and experimental conditions are given in 'Materials and Methods'.

48 h of incubation appeared to be linear. On the basis of these findings, 40 h of incubation using 100 µg (except 20 µg in the case of RSV-CAT) of the specific DNA constructs was chosen for all the subsequent transfection experiments.

Differential expression and regulation of chimaeric DNA constructs in primary bovine luteal cell cultures

To investigate ovarian cell specific regulation, chimaeric DNA constructs containing regions of the 5'-flanking sequences of cytochromes P-450_{SCC} and P-450_{17α} were transfected into bovine luteal cell cultures. Table 1 summarizes the relative CAT activities obtained in transfection studies using the cytochrome P-450_{SCC} specific DNA constructs. All of the DNA constructs containing 186 bp or more of the P-450_{SCC} 5'-flanking region exhibited increased levels of CAT activity after forskolin

treatment. The DNA construct containing -186 to $+12$ bp consistently exhibited the highest response to forskolin (up to 6-fold). Further deletion to -100 bp or less completely abolished forskolin responsiveness and basal expression.

In analogy to the above described experiments, a set of transfections was carried out using chimaeric DNA constructs containing -437 , -297 or -100 to $+19$ bp of the 5'-regulatory region of P-450_{17 α} . As shown in Table 1, none of these DNA constructs was expressed at higher levels than the negative control (CAT) and no apparent effect could be attributed to forskolin treatment. To test whether these DNA constructs were non-functional, the construct containing -297 to $+19$ bp was transfected into Y1 cells. This resulted in cells expressing CAT activity sufficient to convert 70% of the substrate, indicating that the DNA construct was fully active.

Table 1. Relative expression of reporter gene DNA constructs after transfection of bovine luteal cells in primary culture

DNA construct	CAT activity (% conversion)	
	- Forskolin	+ Forskolin
SCC-896	8.31	14.3
SCC-465	15.2	26.7
SCC-240	47.3	53.5
SCC-186	15.0	56.8
SCC-100	2.35	3.9
SCC-50	4.40	3.10
RSV-CAT	98.5	98.7
CAT	8.75	9.5
17 α -437	2.0	4.8
17 α -297	4.1	4.2
17 α -100	2.5	2.4

Numbers represent % conversion of [¹⁴C]chloramphenicol to acetylated products and are calculated from one single experiment. Similar responsiveness to forskolin was observed in 2 other experiments. Experimental conditions for transfection and for the CAT assay are given in 'Materials and Methods'.

Discussion

Primary cultures of bovine luteal cells have been shown to respond to LH administration with increased production and secretion of progesterone (Stirling *et al.*, 1990). This treatment also elevated mRNA levels encoding P-450_{SCC}, indicative of either increased gene expression or mRNA stabilization (Simpson & Waterman, 1988; Boggarum *et al.*, 1989). We have demonstrated in this study that forskolin is capable of elevating progesterone production and P-450_{SCC} mRNA levels in primary cultures of bovine luteal cells. Since forskolin is known to act by stimulating adenylate cyclase (Seamon & Daly 1981), the cAMP-dependent second messenger system appears to be involved. However, several studies have indicated that progesterone production can also be increased via the PKC-dependent system (Hansel *et al.*, 1987; Davis *et al.*, 1989; Benhaim *et al.*, 1990). It is unclear to date whether the PKC system also influences P-450_{SCC} mRNA levels in bovine luteal cells. The relative importance of cAMP in the regulation of P-450_{SCC} gene expression therefore remains to be evaluated.

Immunocytochemistry was used to ensure that the cell cultures were homogeneous with respect of P-450_{SCC} content. This is important since non-steroidogenic cells would decrease the net CAT

activity corresponding to P-450_{SCC} gene expression. Moreover, it has become clear that large and small luteal cells are distinct in terms of regulation of progesterone production (Hansel *et al.*, 1987; Wiltbank *et al.*, 1989). The bovine luteal cell cultures used in this study were composed of both types of luteal cells. Immunocytochemistry using a P-450_{SCC}-specific antibody demonstrated that small and large luteal cells contain comparable amounts of P-450_{SCC} protein. Moreover, the cell cultures appeared to be free of non-steroidogenic cells such as fibroblasts or endothelial cells. Taken together, it appears that primary cultures of bovine luteal cells provide a suitable system to study cAMP-regulated gene expression of P-450_{SCC}.

In this study we report for the first time a transfection protocol for primary cultures of bovine luteal cells. Electroporation appears to be a powerful technique to introduce chimaeric DNA constructs into these cells and permits study of the molecular mechanisms underlying regulation of gene expression throughout the ovarian cycle. Transfection experiments using the bovine luteal cell system provide evidence that sequences of the P-450_{SCC} regulatory region are capable of conferring basal expression and cAMP responsiveness to the CAT reporter gene. Moreover, these *cis*-acting elements appear to be located between -186 and -100 bp upstream of the transcriptional start site of the P-450_{SCC} gene. These results are in good agreement with the findings of similar experiments using the Y1 mouse adrenal tumour cell line (Ahlgren *et al.*, 1990). Sequence analysis of this P-450_{SCC} specific region (-186 to -100 bp) revealed no obvious homology to the cAMP responsive element (CRE) reported by Montminy *et al.* (1986) in the somatostatin gene. It is therefore possible that cAMP regulation of steroidogenic enzymes is carried out by distinct *cis*- and *trans*-acting factors.

The results from transfection studies using the P-450_{17 α} -specific DNA constructs are consistent with the fact that bovine luteal cells do not express P-450_{17 α} . The regulation of the P-450_{17 α} reported gene constructs is therefore similar to that observed for the endogenous gene. This is in contrast to the fact that in Y1 mouse adrenal tumour cells P-450_{17 α} reporter gene constructs are highly expressed, although the endogenous gene is not (Lund *et al.*, 1990). This observation indicates that the mechanisms whereby Y1 cells and bovine luteal cells regulate gene expression of P-450_{17 α} are different.

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