Journal of Steroid Biochemistry, Vol. 11, pp. 1507 to 1512
Pergamon Press Ltd 1979. Printed in Great Britain

EFFECTS OF CYCLIC AMP AND OF 2-MERCAPTO-1-(β-4-PYRIDETHYL)BENZIMIDAZOLE ON COMPOSITION OF STEROIDS SECRETED BY A TESTICULAR TUMOUR CELL LINE

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(*Received* 17 *January* 1979)

SUMMARY

We studied the effect of cyclic AMP (cAMP) on steroidogenesis in a mouse Leydig cell tumor line (I-10), known to secrete exclusively progesterone (P) and 20α-dihydroprogesterone (20α-H_2P). Radioimmunoassays that distinguish these two steroids were used. Total steroidogenesis was stimulated by cAMP in a dose-dependent manner over the range tested (10^{-6}–10^{-3} M). Up to 2×10^{-5} M cAMP, progesterone constituted 11–13% of the secreted progestins; at higher concentrations of cAMP (10^{-4}–10^{-3} M), the P/(P + 20α-H_2P) ratio progressively increased (37% at 10^{-3} M), but the incremental progestin secretion consisted of 50% progesterone throughout this range. The change in progestin profile occurred within less than 45 min. 2-Mercapto-1-(β-4-pyridethyl)benzimidazole (MPB) reduced basal steroidogenesis, progesterone secretion being more severely affected than that of 20α-H_2P. MPB inhibited cell growth and noncompetitively inhibited cAMP-dependent protein kinase activity in the cytosol of I-10 cells. In a faster-growing variant of I-10, higher concentrations of exogenous cAMP were required to exert similar effects on steroidogenesis, and MPB was less effective in suppressing cell growth. The possibility is discussed that cAMP may accelerate an active process of progesterone release, thus minimizing the intracellular exposure of the hormone to 20α-hydroxysteroid dehydrogenase, and that MPB antagonizes cAMP at a site influencing both steroid synthesis and release.

INTRODUCTION

Shin[1] described a steroid-producing cell line, derived from a mouse Leydig cell tumor, that secreted progesterone (P) and its reduced derivative, 20α-dihydroprogesterone (20α-H_2P), but no androgens. Release into the medium of total progestins, estimated by absorption at 240 nm, was not affected by gonadotropins, but was stimulated by exogenous cyclic AMP (cAMP) even at low concentrations of the nucleotide.

With the availability of specific radioimmunoassays for progesterone and 20α-H_2P [2], we were able to examine the effect of cAMP on the secretion of individual steroids by the original cell line and by a faster-growing derivative (I-10B) obtained in our laboratory. It was anticipated that changes in steroidogenesis may be associated with modified growth characteristics, since cyclic nucleotides have been implicated in both processes [3, 4].

In addition to the effects of exogenous cyclic AMP on these cells, we examined the effects of 2-mercapto-1-(β-4-pyridethyl)benzimidazole (MPB), a compound shown to inhibit a wide variety of cell functions [5–14], and shown to inhibit protein kinase activity in homogenates of ovary and uterus from immature rats [M. Lahav, unpublished observations].

MATERIALS AND METHODS

Cell lines and incubation conditions

The I-10 cell line (Leydig cell testicular tumor, mouse, CCL 83) was obtained from the American Type Culture Collection, Rockville, Maryland. The cells (10^5 per dish) were seeded in 15 × 60 mm Petri dishes, incubated at 37°C in 90% air and 10% CO_2 in Ham's F-10 medium (3 ml per dish) supplemented with 15% horse serum and 2.5% fetal serum, and replated approximately once a week. Medium was changed every other day, and always on the day preceding an experiment. Experiments were started by replacing the overnight medium by 2 ml fresh medium containing the indicated additions. Two groups of dishes (three or more per group, containing cells in the late exponential phase) were allocated to each treatment. At the end of the incubations, the medium was removed and stored at −20°C until required for steroid analysis.

I-10B cells were obtained by growing I-10 cells for a long time in the stationary phase. After a few repeated transfers at 16–20 day intervals, a cell

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population with increased growth rate was obtained (doubling time approximately half that of I-10). I-10B was then maintained at the same conditions as I-10.

MPB solution was freshly prepared for each experiment: a solution of 40 mg/ml in dimethylsulfoxide (DMSO) was diluted 1:1000 in medium. The incubation medium of all cultures contained 0.1% DMSO.

Cyclic AMP determination

The cells were rinsed once with 3 ml cold phosphate-buffered saline, pH 7.4 (PBS) and frozen by pouring liquid nitrogen into the dish. The cells were then collected in ice-cold distilled water, pooling the contents of each set of three dishes, and lyophilized. The lyophilate was resuspended in sodium acetate buffer (0.5 M, pH 4), homogenized at 4°C and a sample was taken for protein determination: the remainder was added to an equal volume of boiling acetate buffer, and, after 3 min at 100°C, the homogenate was chilled and centrifuged (12,000 *g*, 15 min) and the supernatant was assayed for cAMP according to Gilman[15].

Determination of progestins

Medium from each set of dishes was pooled, and extracted with 10 vol redistilled ether. The extracts were dried under nitrogen and assayed for progesterone and 20α-H_2P by radioimmunoassay [2]. For estimation of intracellular progestins, the cells were washed three times with cold PBS (2.5 ml/dish/wash) and frozen with liquid nitrogen. For each treatment, cells from two sets of 15 dishes each were collected in ice-cold distilled water, lyophilized and resuspended in 1.3 ml sodium acetate buffer (0.05 M, pH 4). After homogenization at 4°C, samples were taken for protein determination and the homogenate was extracted by 15 vol. ether and analyzed by the procedure cited above [2].

Protein was determined by the method of Lowry *et al.*[16].

Protein kinase was determined by a modification of the procedure of Kuo and Greengard[17]. I-10 cells ($1.5–2.5 \times 10^8$) were collected from seven-day-old cultures. All subsequent procedures were carried out at 4°C. The cells were washed with PBS, resuspended in 4.5 ml buffered sucrose (50 mM Tris, 1 mM $MgCl_2$, 0.25 M sucrose, pH 7.6) and homogenized with a teflon-pestle glass homogenizer (medium motor speed, 13 strokes). The homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant was centrifuged again at 150,000 *g* for 45 min. The cytosol thus obtained was dialized (with two changes) against a buffer containing 20 mM potassium phosphate, 10 mM magnesium acetate, 10 mM, NaF and 0.3 mM EGTA, pH 7.5. The reaction mixture contained in the same buffer (total volume 0.2 ml), 2 μmol theophylline, 100 μg histone, 20 nmol γ^{32}-ATP (3×10^5 c.p.m.) 0 to 0.4 nmol cAMP, 0.1 μl DMSO, 0 or 4 μg MPB and 50 μl cytosol (8–15 μg protein). The assay [17] was run in triplicates. The results were corrected by subtracting the radioactivity measured in histone-free incubation mixtures.

Materials. 2-Mercapto-1-(β-4-pyridethyl)benzimidazole (MPB) was a generous gift of Midland-Yorkshire Tar Distillers Ltd., Oldbury, Warley, Worcs., England. Powdered F-10 medium, horse serum and fetal calf serum were purchased from Grand Island Biological Company. Tritiated steroids [1,2,6,7-^{3}H]-progesterone (80–100 Ci/mmol) and [1,2-^{3}H]-20α-hydroxypregn-4-en-3-one (40–60 Ci/mmol) were products of New England Nuclear Corp., Boston. Cyclic AMP was obtained from Sigma and 20α-dihydroprogesterone and progesterone were purchased from Ikapharm, Jerusalem.

RESULTS

Effect of exogenous cyclic AMP on progestin secretion by I-10 and I-10B cells

The two cell populations studied differed in their growth rate, the doubling time during the exponential phase being 1.5 (I-10) and 0.8 (I-10B) days (Fig. 2, control curves). Cells of both lines (approximately 2×10^6 cells/dish) were incubated for 7 h with various concentrations of cAMP, and progesterone and 20α-H_2P were determined in the culture medium (Fig. 1). The lowest concentration of cAMP used (10^{-6} M) was sufficient to double the amount of progestins secreted; with the highest cAMP concentration (10^{-3} M), which caused a 100-fold or greater stimulation, the rate of steroidogenesis had not yet reached a plateau, though there was a suggestion that the increase was biphasic (Fig. 1). In I-10, the percentage of progesterone in the steroid secretion remained stable and equal to the control (11–13%) over the range of 10^{-6} to 2×10^{-5} M cAMP, but increased progressively with cAMP concentrations from 10^{-4} M (20%) to 10^{-3} M (37%). In cAMP-treated I-10B cells, the same biphasic stimulation of steroidogenesis and the same preferential production of progesterone at high concentrations occurred; however, to obtain comparable effects in the two cell populations, approximately three-fold higher concentrations of cAMP were required in the faster-growing cells.

The time-course of the shift to preferential progesterone secretion in I-10 cells at 10^{-3} M cAMP is shown in Table 1. The rate of overall steroidogenesis increased with time over the 4 h period of the experiment, but the percentage of progesterone in the secreted steroids was essentially the same (33–38%) after 45 to 240 min incubation, suggesting that the cAMP-induced change in the steroid profile has a short latency.

In another experiment (Table 2), cAMP was found to induce a 30-fold increase in intracellular progestin content, while progestin accumulation in the medium increased 130-fold. The steroid content of the cells was negligible compared to that in the medium.

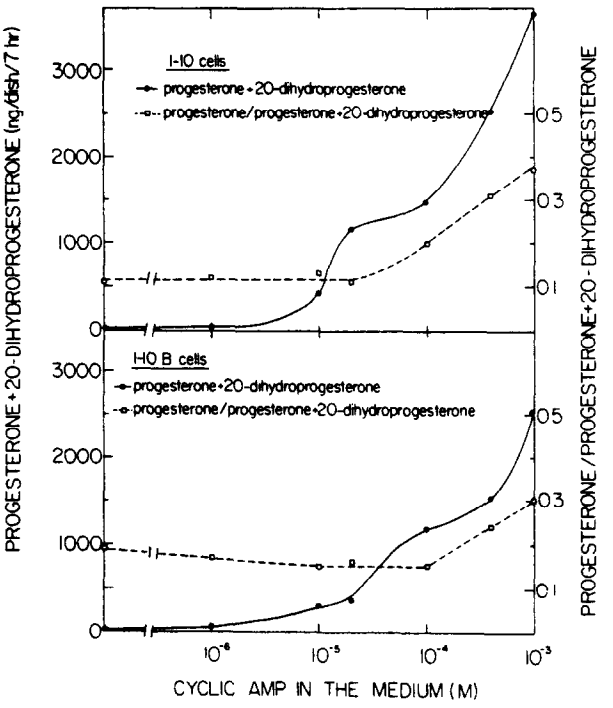

Fig. 1. Effect of exogenous cAMP on the secretion of progesterone and 20α-dihydroprogesterone in I-10 and I-10B cells. Closed circles, total progestins secreted (ng/dish/7 h). Open squares, ratio of progesterone to total progestins.

Effect of MPB on cell growth and progestin synthesis

MPB inhibited growth of I-10 and I-10B cells in a dose-dependent manner, but was less effective in I-10B (Fig. 2); in I-10 cells growth was completely arrested by 20 μg/ml MPB, while in the faster-growing population even 40 μg/ml was only partly effective.

As shown in Fig. 3, steroidogenesis was inhibited by MPB (40 μg/ml) in I-10 cells incubated for 2 or 5½ h. The effect was more marked after the longer incubation period, and progesterone production was suppressed more than that of its metabolite. During the last 3½ h (values obtained by subtraction), MPB inhibited steroid accumulation by 85% and reduced the percentage of progesterone to 6%, compared to 17% in the controls.

Table 1. For each treatment group, two pools of medium, each derived from three culture dishes, were analyzed in triplicate; shown are mean values ± range

Cyclic AMP added to the medium	Incubation time (min)	P + 20α-H_2P ng/dish	P/P + 20α-H_2P
0	240	18.0 ± 3.2	0.14 ± 0.04
10^{-3} M	45	46.4 ± 4.3	0.33 ± 0.01
10^{-3} M	90	230.3 ± 40	0.38 ± 0.01
10^{-3} M	240	1228.0 ± 180	0.33 ± 0.02

Table 2. The effect of cyclic AMP on cell content and secretion into the medium of progesterone (P) and 20α-dihydroprogesterone (20α-H_2P) by I-10 cells

Cyclic AMP added to the medium	Progestins in the medium*		Progestins in the cells*	
	P + 20α-H_2P (ng/dish/4 hr)	P/(P + 20α-H_2P)	P + 20α-H_2P (ng/mg protein)	P/(P + 20α-H_2P)
0	19.9 ± 1.3	0.07 ± 0.01	2.6 ± 0.1 (Approx. 0.36 ng/dish)	0.28 ± 0.02
10^{-3}	2622 ± 101	0.25 ± 0	76.3 ± 25.9 (Approx. 10.7 ng/dish)	0.20 ± 0.04

* Mean (± range) of duplicate samples, each obtained from pooled cells from 14 dishes (140 μg protein per dish).

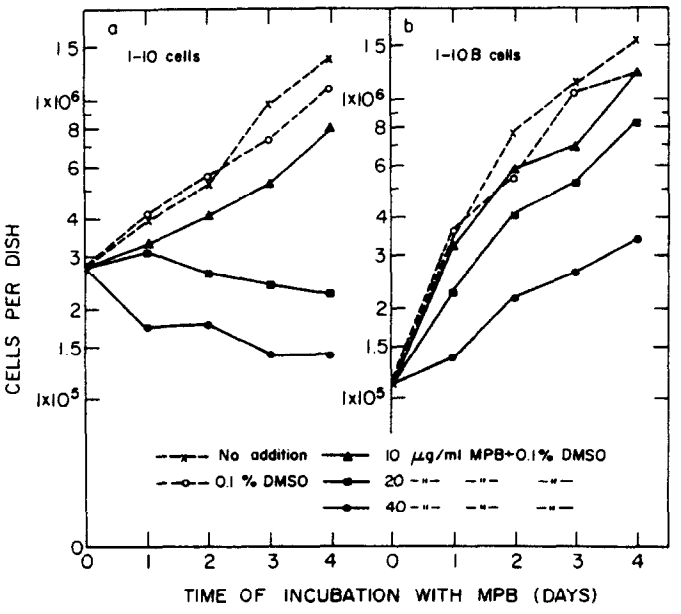

Fig. 2. Effect of various concentrations of MPB on growth of I-10 and I-10B cells.

Effect of MPB on intracellular cAMP and protein kinase activity

Intracellular cAMP was determined in I-10 cells incubated for 4 h with MPB or DMSO (Table 3). MPB slightly reduced the intracellular level of cAMP, while in the solvent control (DMSO) the level was somewhat increased. The difference between the two groups (43% inhibition) was statistically significant ($P < 0.05$).

The results shown in Fig. 4 are representative of those obtained in many experiments in which the effect of MPB on protein kinase activity was tested. At 10^{-5} M, cAMP stimulated protein kinase activity in dialyzed cytosol of I-10 cells 2.5–3-fold. MPB inhibited cAMP-dependent enzyme activity while having a negligible effect on basal protein kinase activity. Inhibition of cAMP-dependent activity was less at higher concentrations of cAMP, but complete reversal was not achieved.

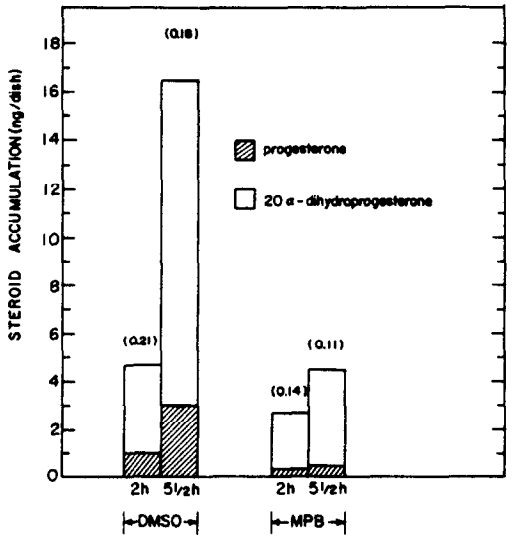

Fig. 3. Effect of MPB (40 μg/ml) on progestin release into medium by I-10 cells during 2 and 5½ h incubations. For each treatment group, two samples of medium were analyzed, each derived from three dishes. Duplicates differed by 4–19% from their means. Numbers over bars show ratio of progesterone total progestins.

Table 3. Effect of MPB and DMSO on intracellular cAMP in I-10 cells

Addition to the medium	Incubation time (min)	Cyclic AMP (%)
—	0	100
0.1% DMSO	240	134 ± 17
40 μg/ml MPB	240	77 ± 9*

* Difference from DMSO-treated cells: $P < 0.05$.

For each treatment, four independent determinations were done, each on a pool of three dishes. Cyclic AMP content after 4 h incubation is expressed as a percentage of intracellular cAMP concentration at zero time in dishes of the same set. The mean intracellular level of cAMP at time zero was 9.8 ± 1.8 pmol/mg protein. Results are presented as means ±SEM.

DISCUSSION

Shin[1] has shown that the testicular tumor cell line I-10 secretes progesterone and 20α-dihydroprogesterone, but no androgens. This author suggested that a loss of 17α-hydroxylase, concomitant with a rise in 20α-hydroxysteroid dehydrogenase (20α-SDH), results in channelling the progesterone formed to a "dead-end" metabolite. A spontaneous rise of

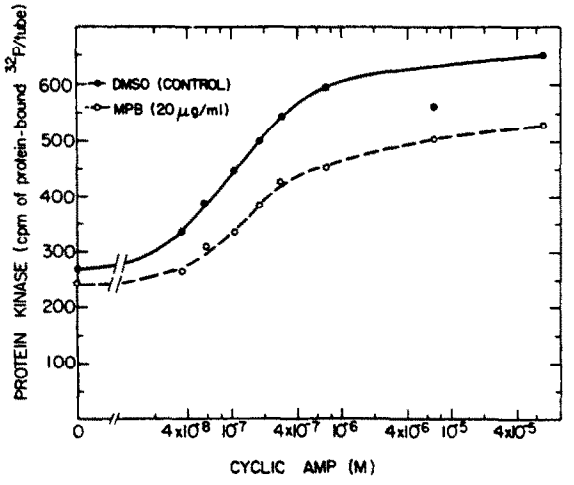

Fig. 4. Effect of MPB on the activity of protein kinase in dialyzed I-10 cytosol. The specific activity of the γ-^{32}P-ATP used was 15 c.p.m./pmol, and the blank value for histone-free incubations substracted was 98 c.p.m. 12.1 μg cytosol protein were added per tube.

20α-SDH under culture conditions has been reported for normal Leydig cells [18], an adrenal tumor [19], granulosa cells [20] and functional corpora lutea of pregnancy [21], all or rat origin. In the luteal cells, addition of prolactin to the culture medium inhibited this rise in enzyme activity [21].

Shin[1] also demonstrated that total progestin production, estimated by optical density measurements, was stimulated by low concentrations of exogenous cAMP. Using specific radioimmunoassays for progesterone and 20α-H_2P, we found that cAMP enhanced the accumulation of both steroids, but the effect on progesterone production was more marked. The threshold cAMP concentration for the change in progestin profile was high (10^{-4} M) compared to the minimal concentration that affected total steroidogenesis (10^{-6} M). The metabolic shift favoring progesterone over 20α-H_2P production must have a short latency, since the percentage of progesterone in the secreted products was the same at 45–240 min after cAMP addition, though the amount of progestins accumulated increased from 47 to 1230 ng/dish during this period (Table 1). The intracellular progestin content was very small compared to that in the medium (Table 2); thus, the preferential progesterone release reflects a relative reduction in the production of 20α-H_2P rather than preferential retention of this metabolite with the cAMP-treated cells.

To explain the change in the steroid profile, one could suggest that at high rates of progesterone production 20α-SDH becomes saturated and thus limits the rate of 20α-H_2P formation. This interpretation is compatable with our observation that the profile shift occurs when the rate of steroidogenesis is quite high. Alternative possibilities are that cAMP influences some metabolic pathways so as to reduce the availability of NADPH, which is essential for 20α-SDH-catalyzed reduction of progesterone, or that the nucleotide inhibits the activity of 20α-SDH. However, all these hypotheses predict that with further stimulation of steroidogenesis, progesterone should constitute an increasing percentage of the incremental progestin secretion, eventually approaching 100%. This was not observed. On the contrary, when steroidogenesis increased from 1150 to 3630 ng/dish by increasing cAMP concentration from 2×10^{-5} to 10^{-3} M, the percentage of progesterone in the incremental secretion remained approximately constant (50%) throughout this range. Thus, the suggestion that 20α-SDH becomes limiting with high concentrations of cAMP does not satisfactorily fit our data.

The possibility remains that cAMP facilitates the release of progesterone, and thus reduces its exposure to intracellular metabolism prior to its secretion. Steroid hormones are usually assumed to leave the steroid-producing cell by simple diffusion [see references 22 and 23 for discussion]. Some recent observations, however, suggest that a more complex mechanism of steroid release may exist, involving the formation of secretory granules and exocytosis [23, 24]. If this is true, then the possibility should be considered that this step is subject to regulation by cAMP.

The effects of MPB on progestin production were opposite to those of added cAMP: basal steroidogenesis was suppressed by MPB and the secretion of progesterone was reduced to a greater extent than that of 20α-H_2P (Fig. 3). MPB slightly reduced endogenous cAMP (Table 3) and inhibited the cAMP-dependent activity of protein kinase of I-10 cytosol (Fig. 4). At increasing concentrations of exogenous cAMP the protein kinase inhibition was attenuated but not abolished.

MPB bears some structural resemblance to purine nucleotides, and this structural analogy may underlie its interference with protein kinase activation by cAMP. A causal relation may exist between this interference and the effects of MPB on progestin secretion, interferon action [10, 25], virus production [8, 26], sodium transport in the toad bladder [14, 27], and induction of certain hepatic enzymes [11, 28].

The responses of I-10B, the faster-growing cell population, resembled that of I-10 qualitatively, but not quantitatively: for obtaining an equivalent effect on steroidogenesis, higher concentrations of cAMP (approximately 3-fold) were required in I-10B cell than in I-10. The faster growth rate of I-10B may also be a reflection of reduced effectiveness of cAMP in this cell population, since there is evidence that specific stages of the replication cycle are impeded by exogenous and endogenous cAMP [4].

Acknowledgements—We thank Mrs. Guenia Acevado for her able technical assistance, and Dr. Fortune Kohen for the supply of antibodies to the progestins. This work was supported by the Ford Foundation and the Population Council, New York. H.R.L. is the Adlai E. Stevenson III Professor of Endocrinology and Reproductive Biology at the Weizmann Institute of Science.

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