J. Steroid Biochem. Molec. Biol. Vol. 41, No. 2, pp. 145–150, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

EFFECT OF A NADPH GENERATING SYSTEM ON THE STEROIDOGENIC RESPONSE IN RAT LUTEAL CELLS

PAULA STEIN and MARTA TESONE*
Instituto de Biología y Medicina Experimental, Obligado 2490 (1428), Buenos Aires, Argentina

(Received 6 June 1991)

Summary—The effect of a NADPH generating system (NADPH-GS) on the function of rat luteal cells was studied. Cells were obtained from pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) primed immature rats and further incubated with a NADPH-GS. This system produced an increase in progesterone production and maximal stimulation was achieved at 1 mM $NADP^+$ (10- to 15-fold). This effect was enhanced by addition of luteinizing hormone (LH 0.25 nM) to the incubation medium. On the contrary, insulin (2 nM) inhibited the effect observed with the NADPH-GS. The conversion of progesterone into 20α-hydroxy-progesterone was not responsible for the changes observed. To analyze the site of NADPH action, pregnenolone and progesterone were measured using two inhibitors of steroid biosynthesis: aminoglutethimide and cyanoketone. The results confirm the specific site of action of NADPH at the mitochondrial conversion of cholesterol to pregnenolone. The effect of NADPH-GS was also observed in cultured purified luteal cells suggesting that the action of NADPH could be mediated by a free entry of the cofactor across the luteal cell plasma membrane. It can be concluded that the addition of NADPH improves the luteal cell incubation conditions and contributes to understanding the regulatory action of LH and insulin on the ovarian steroidogenic process.

INTRODUCTION

Classical studies have amply demonstrated that reduced NADPH is an essential cofactor in the gonadal biosynthesis of steroid hormones [1, 2]. Major generators of this cofactor in the gonads are the dehydrogenases of the citric acid and pentose shunt systems. There is an electron transfer system for steroids in mitochondria and microsomes whose first step is NADPH. It has been reported that the rate of *in vitro* steroid biosynthesis in the gonads and adrenal gland may be increased by the addition of a NADPH generating system (NADPH-GS) [3–7].

The proposed mechanisms by which luteinizing hormone (LH) stimulates the steroidogenic pathway in corpus luteum are: (a) an increase in the amount of NADPH; (b) activation of cholesterol ester hydrolase; (c) an increase in the availability of cholesterol by promoting its transport into mitochondria; and (d) activation of one of the components of the cholesterol side-chain cleavage enzyme system in the mitochondria [8]. Luteal cells obtained from superovulated rats after gonadotropin treatment are only mildly sensitive to LH stimulus [9]. This observation may be interpreted as a possible refractoriness of the cells after *in vivo* human chorionic gonadotropin (hCG) stimulation, a depletion of the cholesterol employed as a substrate for steroidogenesis or as a requirement of NADPH as an essential cofactor for steroid production. In order to explore the latter hypothesis, the stimulatory action of enzymatically generated NADPH on luteal progesterone synthesis and the effect of this cofactor on the action caused by two ovarian tropic hormones, LH and insulin [10–13], were evaluated.

EXPERIMENTAL

Materials

Pregnant mare serum gonadotropin (PMSG) and hCG used for superovulation were kindly donated by Elea Laboratories (Buenos Aires, Argentina). [1,2,6,7-^{3}H(N)]progesterone (98.3 Ci/mmol), [1,2-^{3}H(N)]20α-hydroxy-progesterone (45.6 Ci/mmol), [7-^{3}H(N)]pregnenolone (19.1 Ci/mmol) and [2,8-^{3}H]adenosine 3′,5′-cyclic phosphate (cAMP, 34.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA).

*To whom correspondence should be addressed.
Trivial names: 20α-hydroxy-progesterone, 20α-hydroxy-4-pregnen-3-one; 25-hydroxy-cholesterol, 5-cholesten-3β-25-diol.

Unlabelled progesterone, 20α-hydroxy-progesterone, pregnenolone, cAMP, Hepes, deoxyribonuclease I (DNase I), bovine serum albumin fractions V (BSA), D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase and $NADP^+$ were from Sigma Chemical Co. (St Louis, MO). Dulbecco's Modified Eagle Medium with 4.5 g glucose/l (DMEM), Ham's F-12 nutrient medium (F-12) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY). 25-Hydroxy-cholesterol, cyanoketone and aminoglutethimide were obtained from Steraloids (Wilton, NH). Collagenase (153 IU/mg) was from Worthington Biochemical Co. (Freehold, NJ). Human LH (hLH: 7.2 IU/μg) was kindly supplied by Hormone Laboratory, Aker Hospital (Oslo, Norway). Insulin was the generous gift of Eli Lilly Co. (Indianapolis, IN).

Preparation of collagenase-dispersed luteal cells from superovulated rats

Female Sprague–Dawley rats, 23–25 days old, fed Purina rat chow *ad libitum* and kept in an air-conditioned atmosphere, were injected with PMSG (25 IU/rat) and 48 h later with hCG (25 IU/rat). Experiments were carried out 7 days after PMSG administration. Luteal cells from luteinized ovaries were isolated basically as described previously [14]. In brief, ovaries were finely minced in DMEM–F12 (1:1 v/v), containing Hepes 10 mM, BSA 0.1% (DMEM–F12-Hepes). Enzymatic digestion was made using 1 ml per ovary of DMEM–F12-Hepes plus collagenase (0.1%) and DNase I (0.008%). The tubes were shaken at 37°C for 40 min. The suspension was passed through Nytex and centrifuged at 250 *g* for 10 min. The pellet was washed twice and resuspended in the same medium. The viability of the cells was in the 90% range, as determined by trypan blue staining.

Incubation of luteal cells

Isolated luteal cells (250,000 cells/0.5 ml) from superovulated rats were incubated under constant shaking at 37°C for 3 h with different stimuli.

The NADPH-GS consisted of: 1 mM $NADP^+$, 1 IU D-glucose-6-phosphate dehydrogenase and 5-mM D-gloucose-6-phosphate [15]. hLH or insulin were added at 10 ng/ml (0.25 nM or 2 nM, respectively); these hormone concentrations produce maximal progesterone stimulation in luteal cells [14]. Other factors tested were: 25 hydroxy-cholesterol (10 μg/ml), aminoglutethimide (1 mM) and cyanoketone (0.5 mM). When used, NADPH was added at 1 mM; this amount was the optimal concentration found, measured as progesterone stimulation.

Incubations were terminated by centrifugating the vials at 800 *g* for 15 min at 4°C and the supernatants were frozen for determination of steroids by radioimmunoassay (RIA) (see below). In some experiments, 0.25 ml aliquots of the supernatants were heated at 100°C for 15 min and stored at −20°C until the determination of cAMP by means of a protein binding competition assay [16].

Luteal cell purification and culture

To purify the luteal cell population, the ovarian cell suspension was layered on a discontinuous density gradient (Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden) at room temperature. Gradients, comprised of 3 ml each of densities 1.020, 1.042, 1.060 and 1.085 g/ml, were layered in a polystyrene culture tube and centrifuged at 600 *g* for 20 min. The cells present between the density layers of 1.020 and 1.042 g/ml were aspirated, resuspended and counted in a hemocytometer. In this preparation no cellular debris or blood cell contamination was evident. Cell viability (>90%) was tested as described above. Purified luteal cells were used for experiments in culture. Cell culture was made as described previously [14]. In brief, plastic 24 multiwells (Falcon) were coated with fetal bovine serum and approx. 500,000 cells in DMEM–F12 containing 0.2% BSA were seeded per well and placed in a culture chamber (5% CO_2 in air at 37°C). After 16 h culture media were changed to remove non-attached cells. Cells were cultured for 3 h with different stimuli. At the end of the incubation period media were removed and frozen at −20°C for progesterone determination by RIA.

Other methods

Luteal cell production of progesterone, 20α-hydroxy-progesterone and pregnenolone was evaluated by RIA, as described previously [9, 14]. RIA was performed directly in suitable dilutions of the incubation media.

All results are given as the mean ±SEM of triplicate incubations. Statistical comparisons were performed by analysis of variance

(ANOVA) [17]. $P < 0.05$ was considered statistically significant. Experiments were repeated at least twice with 8–10 animals per experiment.

RESULTS

The dose–response curve of NADPH-GS is shown in Fig. 1. Maximal progesterone stimulation was attained at 1 mM $NADP^+$. Higher concentrations of the cofactor did not cause any change in the response. In all NADPH-GS concentrations tested, insulin (2 nM) was able to inhibit progesterone induction. On the other hand, LH (0.25 nM) enhanced the effect obtained with different concentrations of NADPH-GS, even the maximal response reached at 1 or 10 mM $NADP^+$. Similar results were obtained using NADPH as cofactor (data not shown), suggesting that this reduced cofactor is produced outside the luteal cell and is able to participate in intracellular events.

To determine whether the stimulatory action of NADPH on progesterone production by luteal cells could reflect a change in the conversion of progesterone to its main catabolite, 20α-hydroxy-progesterone, this steroid was measured in the incubation medium (Fig. 2). It was observed that basal levels of 20α-hydroxy-progesterone were increased by the presence of NADPH-GS, according to a major available substrate. Neither LH nor insulin caused any significant change in the stimulatory effect exerted by NADPH-GS on 20α-hydroxy-progesterone accumulation.

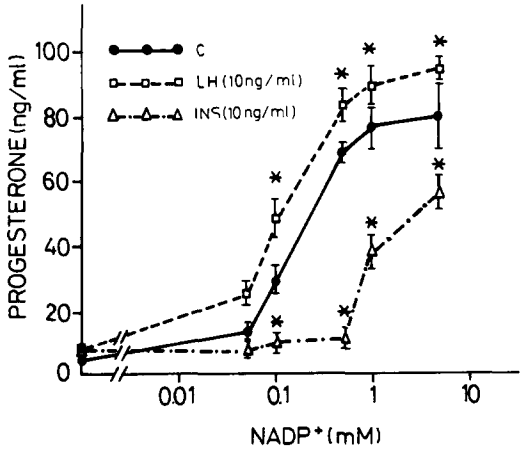

Fig. 1. Effect of increasing doses of NADPH-GS alone (C) or in the presence of LH or insulin (INS) on progesterone production in isolated luteal cells. Cells (250,000 cells/0.5 ml) were incubated at 37°C in DMEM–F12-Hepes for 3 h. At the end of this time, tubes were centrifuged at 800 *g* for 10 min at 4°C and then the supernatant kept frozen at −20°C until progesterone determination by radioimmunoassay. Data are means ± SEM of triplicate incubations. These results are typical of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$) from the corresponding controls.

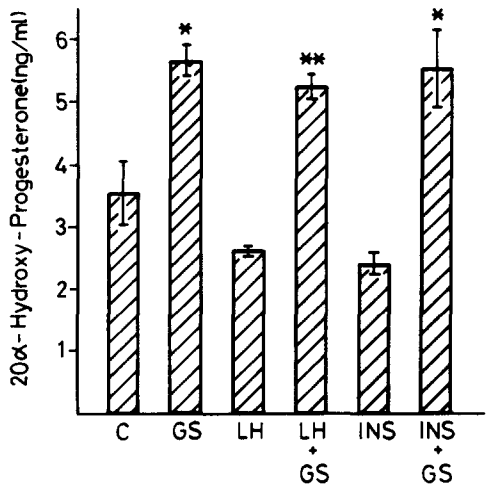

Fig. 2. 20α-Hydroxy-progesterone production by rat luteal cells incubated with NADPH-GS (GS: 1 mM $NADP^+$), LH (0.25 nM), insulin (INS: 2 nM) or combinations of these stimuli. Cells were incubated under the conditions described in the legend to Fig. 1. 20α-Hydroxy-progesterone was measured by RIA. Each bar represents the mean ± SEM of triplicate determinations. Asterisks indicate significant differences (*$P < 0.01$; **$P < 0.05$) from the control.

Several reports [18, 19] described adenosine receptors in rat luteal cells and an increase in the cAMP production as a consequence of *in vitro* adenosine stimulation. To discard a possible interaction between NADPH and adenosine receptors, cAMP accumulation was measured in luteal cell incubations. We observed that only in the presence of LH there was a significant increase (7-fold) in the luteal cell cAMP production over the control value (data not shown).

To permit the analysis of NADPH-GS action on the cholesterol side chain cleavage enzyme, pregnenolone production was measured using two inhibitors of steroid biosynthesis. The first compound was cyanoketone, an inhibitor of 3β-hydroxysteroid oxidoreductase-5-en-isomerase activity *in vitro* and *in vivo* [20, 21]. The second one was aminoglutethimide, an inhibitor of steroid biosynthesis known to act upon cytochrome *P*-450-dependent enzymes in the mitochondrial ovarian fractions [8]. Cells stimulated by NADPH-GS in the presence of aminoglutethimide (1 mM) produced lower amounts of pregnenolone and progesterone than in the absence of the inhibitor. Additionally, as expected, cyanoketone (0.5 mM) reduced progesterone production as a consequence of pregnenolone accumulation (Fig. 3).

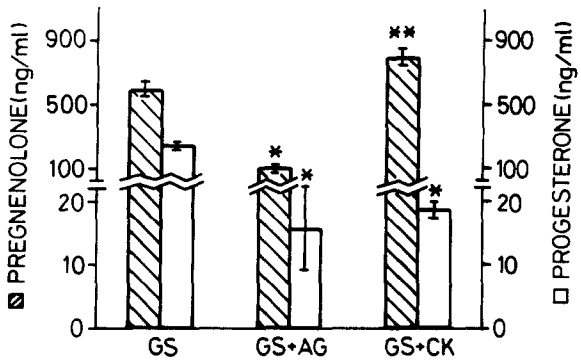

Fig. 3. Progesterone and pregnenolone production by isolated luteal cells during incubation with NADPH-GS (GS: 1 mM $NADP^+$), NADPH-GS plus aminoglutethimide (AG: 1 mM) or NADPH-GS plus cyanoketone (CK: 0.5 mM). Cells were incubated under the conditions described in Fig. 1. Progesterone and pregnenolone were measured by RIA. Each bar represents the mean ± SEM of triplicate determinations. Asterisks indicate significant differences (*$P < 0.01$; **$P < 0.05$) vs NADPH-GS.

To investigate whether the presence of an exogenous steroidogenic substrate could modify the response of luteal cells to the NADPH-GS, cells were incubated with 25 hydroxy-cholesterol. This polar analogue of cholesterol has been shown to serve as a better exogenous substrate than cholesterol in rat luteal cells, presumably because of its ready access to the mitochondrial side-chain cleavage enzyme system [22]. In the presence of 25-hydroxy-cholesterol, NADPH-GS-treated cells showed a similar increase in progesterone output over their respective controls (Fig. 4). However, the addition of LH and 25-hydroxy-cholesterol produced enhanced the maximal response reached with the NADPH-GS, suggesting that the exogenous substrate might be used if the SCC enzyme system is activated by LH.

To confirm the effect of NADPH-GS in a further purified cell preparation, luteal cells were purified by a Percoll gradient and then tested in culture. This offers the advantage of providing an intact cell culture with the ability to attach and spread in culture plates. Figure 5 shows the effect of different stimuli on progesterone production. Cells were cultured in serum-free medium for 16 h. Culture media were changed to remove non-attached cells and cells were cultured for 3 h with NADPH-GS or NADPH. Both significantly increased the amount of progesterone secreted by the cells. This increase was lower than that observed in suspension (4- vs 10-fold), probably as a consequence of the presence of damaged cells in the non-purified cell preparation, allowing better access of NADPH to the enzymes involved in steroidogenesis.

DISCUSSION

These observations extend and confirm previous results showing that addition of

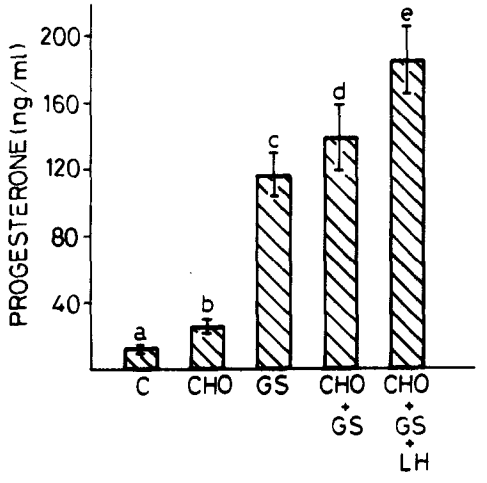

Fig. 4. Effect of 25-hydroxy-cholesterol on the stimulatory action of NADPH-GS. Luteal cells were incubated with 25-hydroxy-cholesterol (CHO; 10 μg/ml), NADPH-GS (GS: 1 mM $NADP^+$) or both. The stimulatory effect of NADPH-GS was amplified in presence of 25-hydroxy-cholesterol plus LH (0.25 nM) but not with 25-hydroxy-cholesterol alone. Incubation procedures and progesterone determinations were carried out as described under Experimental. Each bar represents the mean ± SEM. c, d, and e vs a: $P < 0.01$; e vs c: $P < 0.05$.

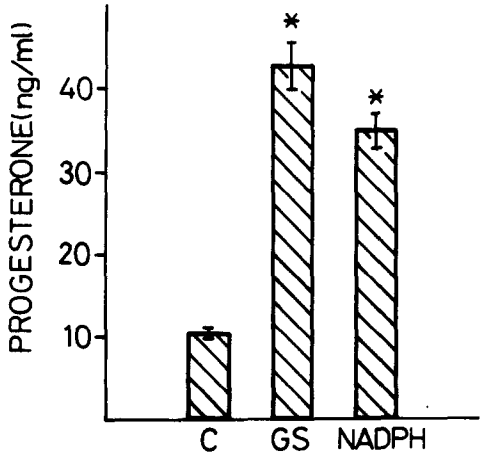

Fig. 5. Influence of NADPH-GS (GS: 1 mM $NADP^+$) and NADPH (1 mM) on progesterone production by Percoll-purified luteal cells. Cells (500,000/well) were cultured in serum-free medium (DMEM–F12) under an atmosphere of 5% CO_2 in AIR for 16 h. Culture media were changed to remove non-attached cells and cells were cultured for 3 h with the different stimuli. Media were kept frozen at −20°C until determination of progesterone by RIA. Each bar represents the mean ± SEM of triplicate cultures. Statistical comparisons were made in relation to control values (*$P < 0.01$).

NADPH-GS or NADPH can stimulate hormone synthesis in steroidogenic tissue preparations [1–6]. Blankenstein *et al.* [7] showed in isolated Leydig cells that testosterone production is increased when NADPH-GS is added to the incubation medium. We have obtained similar results using collagenase-dispersed rat luteal cells. Maximal progesterone response to a NADPH-GS was attained at 1 mM $NADP^+$. The regulatory effect of insulin on this process was just the opposite as that of LH; this would reflect the different mechanisms involved in the tropic action of both hormones in luteal cells. The inhibitory effect of insulin on the progesterone increase induced by NADPH-GS could be due to a selective stimulation of the NADPH–oxidase enzyme [23]. It also could be due to an activation of fatty acid synthesis, employing NADPH as a cofactor for reductase enzymes [24]. On the other hand, LH has a stimulatory effect on the increased progesterone production obtained with NADPH-GS in the incubation medium. This can reflect the interaction of the hormone with high affinity membrane receptors [25], activation of the adenylate cyclase–protein kinase enzymatic pathway leading to the stimulation of the cholesterol side-chain cleavage enzyme system [8]. LH is also able to increase maximal response to NADPH-GS when exogenous 25-hydroxy-cholesterol is added to the incubation media. Without LH, the addition of 25-hydroxy-cholesterol did not elicit any change in the action of NADPH-GS. This suggests that under these conditions the steroidogenic enzymes capable of being stimulated by LH are the limiting factor in steroid biosynthesis.

The results obtained using steroidogenic inhibitors reveal that the specific site of action of NADPH is at the mitochondrial conversion of cholesterol to pregnenolone, which in turn renders an increase in the production of luteal progesterone.

Interestingly, the NADPH-GS effect is also observed in cultured purified luteal cells, where the presence of collagenase-damaged cells is highly reduced. These data suggest that the action of NADPH could be mediated by a free entry of the cofactor across the luteal cell plasma membrane, as it was proposed in Leydig cells [7]. However, the fact that cultured cells are affected to a lesser extent by external NADPH than cells in suspension could indicate that damaged cells, which are reduced but not totally eliminated in the cultures, would be responsible for the NADPH stimulation.

It can be concluded that the addition of NADPH improves the luteal cell incubation conditions and contributes to understanding the regulatory action of LH and insulin on the ovarian steroidogenic process.

Acknowledgements—We wish to thank Luis Jiménez de Asúa Ph.D. for the stimulating discussions and revision of the manuscript. This paper was supported by grants from TWAS (Third World Academy of Sciences), Antorchas Foundation and CONICET (National Research Council, Argentina).

REFERENCES

1. Hall P. F. and Koritz S. B.: The conversion of cholesterol and 20α-hydroxycholesterol to steroids by acetone powder of particles from bovine corpus luteum. *Biochemistry* **3** (1964) 129–134.
2. Nielson M. H. and Warren J. C.: TPNH generation in the human ovary, *Acta Endocr.* **49** (1965) 58–64.
3. Constantopoulos G. and Tchen T. T.: Cleavage of cholesterol side chain by adrenal cortex. *J. Biol. Chem.* **236** (1961) 65–67.
4. Halkerston I. D. K., Eichhorn J. and Hechter O.: A requirement for reduced triphosphyridine nucleotide for cholesterol side-chain cleavage by mitochondrial fractions of bovine adrenal cortex. *J. Biol. Chem.* **236** (1961) 374–380.
5. Mason N. R., March J. M. and Savard K.: An action of gonadotropin *in vitro*. *J. Biol. Chem.* **237** (1962) 1801–1806.
6. Savard K., Marsh J. M. and Howell D. S.: Progesterone biosynthesis in luteal tissue: role of nicotinamide adenine dinucleotide phosphate and NADP-linked dehydrogenases. *Endocrinology* **73** (1963) 554–563.
7. Blankenstein M. A., Van Woerkom-Blik A., Janszen F. H. A. and Van Der Molen H. J.: Effect of cofactors and luteinizing hormone on testosterone production by rat testis preparations. *J. Steroid Biochem.* **7** (1976) 445–450.
8. Mori M. and Marsh J. M.: The site of luteinizing hormone stimulation of steroidogenesis in mitochondria of the rat corpus luteum. *J. Biol. Chem.* **257** (1982) 6178–6183.
9. Ladenheim R. G., Tesone M. and Charreau E. H.: Insulin action and characterization of insulin receptors in rat luteal cells. *Endocrinology* **115** (1984) 752–756.
10. Richards J. S. and Bogovich K.: Effects of human chorionic gonadotropin and progesterone on follicular development in the immature rat. *Endocrinology* **111** (1982) 1429–1438.
11. McNatty K. P., Hudson N., Gibb M., Ball K., Fannin J., Kieboom L. and Thurley D. C.: Effects of long term treatment with LH on induction of cyclic ovarian activity in seasonally anoestrous owes. *J. Endocr.* **100** (1984) 67–73.
12. Adashi E. Y., Resnick C. E., D'Ercole A. J., Svoboda M. E. and Van Wyk J. J.: Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Rev.* **6** (1985) 400–420.
13. Poretsky L. and Kalin M. F.: The gonadotropic function of insulin. *Endocrine Rev.* **8** (1987) 132–141.
14. Saragueta P., Krimer A. R. D., Charreau E. H. and Tesone M.: Insulin regulation of steroidogenic activity in rat culture luteal cells. *J. Steroid Biochem.* **32** (1989) 393–397.

15. Calvo J. C., Valle L. B. S., Barañao J. L. S., Tesone M. and Charreau E. H.: NADPH generating enzymes in Leydig cells from diabetic rats. *Horm. Metab. Res.* **11** (1979) 161–164.
16. Brown B. L., Albano J. D. M., Ekins R. P. and Sgherzi A. M.: A simple and sensitive saturation assay for the measurement of 3′:5′-cyclic monophosphate. *Biochem. J.* **121** (1971) 561–562.
17. Winer B. J.: *Statistical Principles Experimental Design.* McGraw-Hill Book, New York (1971).
18. Billig H., Thelander H. and Rosberg S.: Adenosine receptor-mediated effects by nonmetabolizable adenosine analogs in preovulatory rat granulosa cells: a putative local regulatory role of adenosine in the ovary. *Endocrinology* **122** (1988) 52–61.
19. Soodak L. K., Musicki B. and Behrman H. R.: Selective amplification of luteinizing hormone by adenosine in rat luteal cells. *Endocrinology* **122** (1988) 847–854.
20. Neville A. M. and Engel L. L.: Inhibition of steroid Δ_5-isomerase of the bovine adrenal gland by substrate analogues. *Endocrinology* **83** (1968) 873–876.
21. Goldman A. S.: Inhibition of 3β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni by various estrogenic and progestinic steroids. *J. Clin. Endocr. Metab.* **27** (1967) 320–324.
22. Toaff M. E., Schleyer H. and Strauss III J. F.: Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology* **111** (1982) 1785–1790.
23. Mukerjee S. P. and Lynn W. S.: Reduced nicotinamide adenine dinucleotide phosphate oxidase in adipocyte plasma membranes and its activation by insulin. *Archs Biochem. Biophys.* **184** (1977) 69–76.
24. Stumpt P. K.: Metabolism of fatty acids. *A. Rev. Biochem.* **38** (1969) 159–212.
25. Catt K. J., Tsuruhara T. and Dufau M. L.: Gonadotropin binding sites of the rat testis. *Biochim. Biophys. Acta* **279** (1972) 194–201.